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DETECTION, ISOLATION AND CHARACTERIZATION OF MARINE METHANOTROPHS

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### Declaration

The work presented in this thesis is the result of original research conducted by myself under the supervision of Dr J.C. Murrell. In the instances where others have contributed to the work, specific references have been made.

None of the information contained in this thesis has been used in any previous application for a degree.

Veronica McGowan

## Summary

In order to investigate the occurrence of methanotrophs in the marine environment, representative organisms have been isolated from seawater. As traditional methods for isolating and cultivating methanotrophs were found to be unsuccessful when applied to the marine environment, new techniques for methanotroph isolation were developed. These were used to enrich for methanotrophs in several marine areas.

Two bacteria isolated from marine areas have been extensively characterized. Both isolates appear to be typical of Type I methanotrophs and have an absolute requirement for sodium chloride. The nitrogen assimilation of the isolates was studied in detail. Both isolates appear to have unusual nitrogen assimilation pathways.

A method for detecting methylotrophs and methanotrophs without cultivation has also been developed. The polymerase chain reaction has been adapted to amplify DNA sequences specific to methylotrophs and methanotrophs. This has been used to detect amplify DNA sequences in DNA extracted from water samples from a range of fresh water and marine environments. The methods has been demonstrated to be specific for methylotrophic and methanotrophic DNA. DNA from non-methylotrophic bacteria was not amplified. Initial studies on the application of this method for enumeration has also been studied.

An investigation into the occurrence of methanotrophs in the Southern Ocean was also carried out. Methanotrophic activity was examined in two areas of the Southern Ocean. This was compared with the methane concentration in the water and total bacterial activity.



## Abbreviations

ADH	alanine dehydrogenase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CTD	conductivity, temperature, depth. Equipment for collecting water samples (see section 2.12)
CTP	cytidine triphosphate
DAPI	4'6-diamidino-2-phenylindole
DCPIP	dichlorophenol indophenol
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
KDPG	2-keto 3-deoxy 6-phosphogluconate
LB	Luria broth
MDH	methanol dehydrogenase
MMO	methane monooxygenase
pMMO	particulate form of MMO
sMMO	soluble form of MMO
mol% G+C	cytosine plus guanine content of DNA
mox gene	methanol dehydrogenase gene
MPN	most probable number
MS	mineral salts medium (see section 2.1 for details)
	AMS MS + NH <sub>4</sub> Cl
	ANMS MS + NH <sub>4</sub> Cl + KNO <sub>3</sub>
	NMS MS + KNO <sub>3</sub>
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotidephosphate reduced form
N-NEDH	naphthyl ethylene diamine diHCl
nif gene	nitrogen fixation gene
PBS	physiologically buffered saline
PCR	polymerase chain reaction
PEP	phosphoenol pyruvate
PMS	phenazine methosulphate
PPFM	pink-pigmented facultative methylotrophs
ppm	parts per million
ppt	parts per thyousand
POQ	pyrrolo-quinoline quinone
PVPP	polyvinylpolypyrrolidone
RuMP	ribulose monophosphate
SRBS	sheep red blood cells
Taq polymerase	<u>Thermus aquaticus</u> polymerase
<sup>3</sup> TdR	<sup>3</sup> H-methyl] thymidine

## INTRODUCTION

## 1.1 C<sub>1</sub>-utilizing microorganisms

Bacteria that can utilize C<sub>1</sub> compounds, e.g. methane and methanol, as carbon and energy sources are known as methylotrophs. Methylotrophs have been defined as microorganisms 'able to grow at the expense of reduced carbon compounds containing one or more carbon atoms but containing no carbon-carbon bonds' (Anthony, 1982).

### 1.1.1 Occurrence of methane in nature

Reduced carbon compounds are found throughout the environment. Those that can be utilized as sole carbon sources for methylotrophs are described below and include methane, methanol, methylated amines, formaldehyde and formate.

Methane is the most abundant organic gas in the Earth's atmosphere (Cicerone & Oremland, 1988). The concentration of methane in the atmosphere appears to be increasing and levels of methane have risen dramatically during the past 200 years. The increase in methane concentration has been demonstrated in many areas by a variety of measurement techniques, including flame ionization gas chromatography (for example; Blake & Rowland, 1988) and infrared absorption analysis (Rinsland et al, 1985). The rate of increase in methane concentration has been estimated to be 0.016 ppm/yr or 1% per year between 1978 and 1987 (Blake & Rowland, 1988). From the analysis of ice cores, it appears that atmospheric methane has more than doubled in concentration over the last 200 years, from 0.8 ppm to 1.6 ppm (Rasmussen & Khalil, 1984). The authors also reported that atmospheric methane concentrations for the last 3000 years remained constant, between 0.6 and 0.8 ppm, until 200 years ago. The cause of the rise in methane

concentration is unknown although it is likely to be due to human activities (Cicerone & Oremland, 1988). Methane is present in many environments and there have been several studies on methane concentration in different environments. These are summarized in Table 1.1.

Methane is a 'greenhouse gas': by interaction with planetary infrared radiation, methane leads to a warming of the planets surface and near-surface atmosphere and cools the stratosphere through radiative losses to space. Methane also affects global warming through more indirect routes. The oxidation of atmospheric methane to  $\text{CO}_2$  accounts for 6% of the total  $\text{CO}_2$  produced by anthropomorphic sources (Cicerone & Oremland, 1988). Chemical methane oxidation in the atmosphere can also lead to an increase in tropospheric (lower atmosphere) ozone which acts as a greenhouse gas. It is therefore necessary to understand the natural biological and physical processes which control methane flux.

Methane in the environment can be broken down in two ways: by chemical oxidation or by microbial oxidation. The flow of methane in the environment is illustrated in fig. 1.1. Methane in the atmosphere is chemically oxidised to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . This process is initiated by hydroxyl radicals ( $\text{OH}$ ) (not to be confused with the aqueous  $\text{OH}^-$ ) which are formed by the reaction of ozone with ultraviolet light and water vapour. Microbiological methane oxidation is generally thought to be an aerobic reaction, although there is evidence that an alternative anaerobic route exists. Aerobic methane oxidation proceeds via an enzyme pathway where methane is oxidized to methanol, which is then

ENVIRONMENT	METHANE CONCENTRATION	REFERENCE
Mono Lake, California	(surface waters) 0.09-1.29 $\mu\text{M}$ (bottom waters) 35-57 $\mu\text{M}$	Oremland <i>et al</i> / 1987
Big Soda Lake, Nevada	(surface waters) (0.1 $\mu\text{M}$ ) (bottom waters) 53 $\mu\text{M}$	Iversen <i>et al</i> /1987
Lake Washington	(sediment) 40-90 $\mu\text{M}$	Kuivila <i>et al</i> / 1988
Marine sediment, Jutland	(sediment) <90 $\mu\text{M}$	Iversen & Blackburn 1981
Sargasso Sea	1.9-2.7 nM	Jones 1991
Cariaco Basin	(surface waters) 0.012 $\mu\text{M}$ (bottom waters) 14 $\mu\text{M}$	Ward <i>et al</i> / 1987

*Table 1.1: Methane concentrations of various environments.*

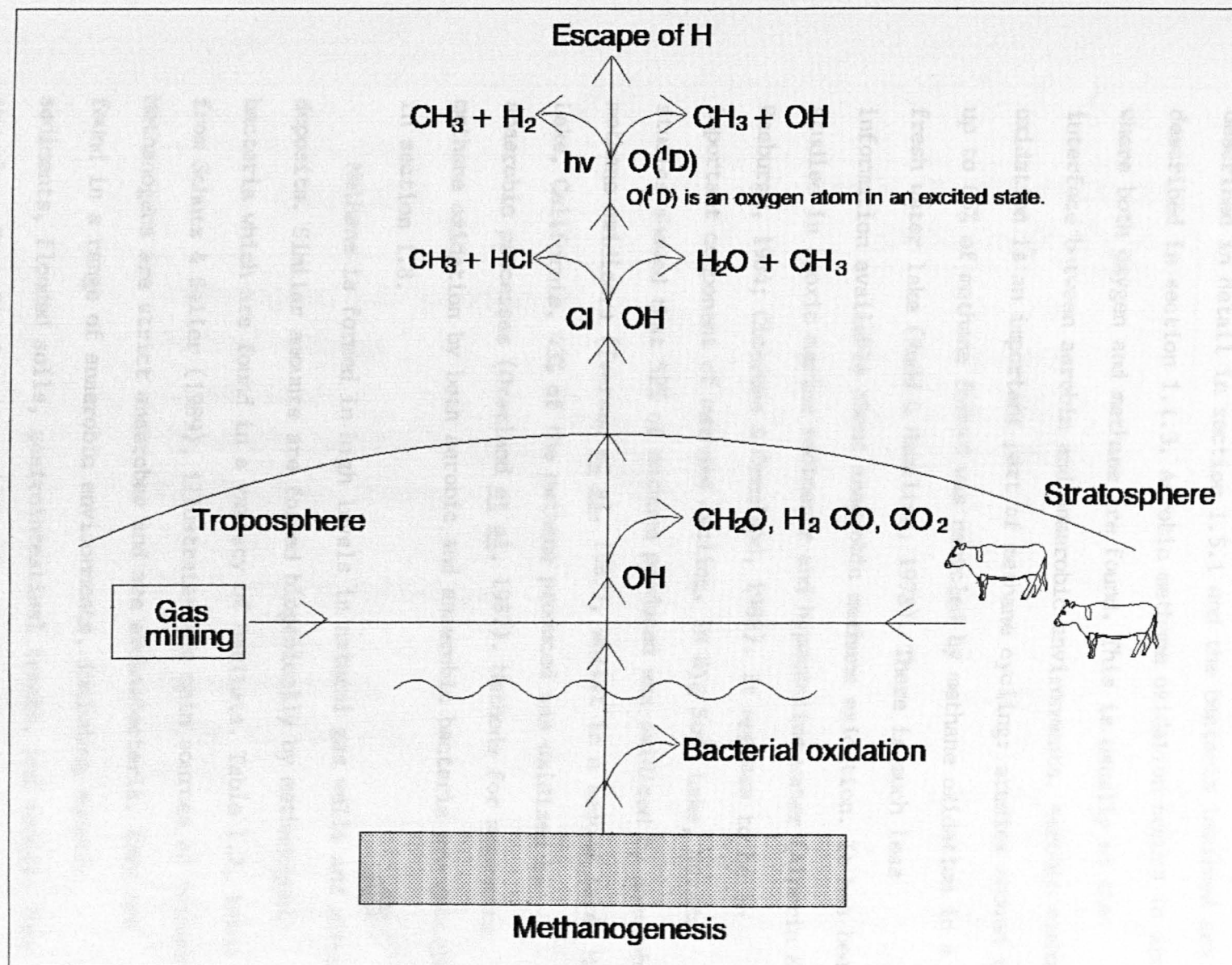


Fig. 1.1 : Schematic illustration of the flow of methane (from Cicerone & Oremland, 1988)

further oxidized to formaldehyde. Formaldehyde is either dissimilated to formate and  $\text{CO}_2$  or is assimilated into the cell. This pathway is described in detail in section 1.5.1 and the bacteria involved are described in section 1.1.3. Aerobic methane oxidation occurs in areas where both oxygen and methane are found. This is usually at the interface between aerobic and anaerobic environments. Aerobic methane oxidation is an important part of methane cycling: studies showed that up to 65% of methane formed was recycled by methane oxidation in a fresh water lake (Rudd & Hamilton, 1978). There is much less information available about anaerobic methane oxidation. It has been studied in anoxic marine sediments and hypersaline water (Alperin & Reeburgh, 1984; Cicerone & Oremland, 1988). It appears to be an important component of methane cycling. In Big Soda Lake, Nevada, studies showed that 52% of methane produced was oxidized by anaerobic methane oxidizers (Iversen et al, 1987), whilst in a second lake, Mono Lake, California, 43% of the methane produced was oxidized by anaerobic processes (Oremland et al, 1987). Methods for measuring methane oxidation by both aerobic and anaerobic bacteria are described in section 1.8.

Methane is formed in high levels in natural gas wells and mineral deposits. Similar amounts are formed biogenically by methanogenic bacteria which are found in a variety of habitats. Table 1.2, taken from Schutz & Seiler (1989), illustrates the main sources of methane. Methanogens are strict anaerobes and are archaebacteria. They are found in a range of anaerobic environments, including aquatic sediments, flooded soils, gastrointestinal tracts, and sewage. They are also often found in extreme environments with high temperatures,

<b>SOURCE</b>	<b>RANGE OF REPORTED CH<sub>4</sub> EMISSION (X10<sup>12</sup> g.yr<sup>-1</sup>)</b>
<b>Waterlogged soils (paddy rice)</b>	<b>70 - 170</b>
<b>Natural wetlands</b>	<b>40 - 160</b>
<b>Landfill sites</b>	<b>30 - 70</b>
<b>Oceans/lakes/other biogenic</b>	<b>15 - 35</b>
<b>Ruminant digestive tract</b>	<b>66 - 90</b>
<b>Termites</b>	<b>2 - 5</b>
<b>Exploration of natural gas</b>	<b>30 - 40</b>
<b>Coal mining</b>	<b>35</b>
<b>Biomass burning</b>	<b>55 - 100</b>
<b>Other nonbiogenic</b>	<b>1 - 2</b>
<b>TOTAL</b>	<b>374 - 717</b>

**Table 1.2 : Range of reported methane emission rates from different sources  
(taken from Schutz & Seiler, 1989)**



high salinity or extremes of pH. Methanogens can utilize a range of substrates to form methane, such as  $\text{CO}_2$ , hydrogen, acetate, formate, methanol, methylated amines and carbon monoxide. These substrates are formed from organic matter by other anaerobic bacteria, and complex bacterial consortia often result. Methanogens are also found in symbiotic relationships with herbivorous animals, where they reside in the gastrointestinal tract and assist in the breakdown of plant polymers such as cellulose. As well as methanogenesis by bacteria, methane is produced by thermocatalytic reactions during petroleum production. Methane from different sources can be distinguished by isotopic analysis (Cicerone & Oremland, 1988). Methanogens preferentially utilize isotopically light carbon, and biogenically produced methane is therefore enriched for  $^{12}\text{C}$ . The methane produced contains only trace levels of ethane and propane. Biogenic methane can be dated by  $^{14}\text{C}$  analysis. Thermogenically produced methane, however, has a low  $^{12}\text{C}$  content, virtually no  $^{14}\text{C}$ , and significant levels of higher alkanes present.

#### 1.1.2 Occurrence of other reduced carbon compounds in nature

Methanol is produced from two natural processes. It is found in low concentrations in the atmosphere where it is produced by oxidation of methane by hydroxyl radicals in the upper atmosphere (Cicerone & Oremland, 1988). It is also formed by the decomposition of plant material such as lignin and pectin. Methanol can be oxidized to formaldehyde by aerobic methylotrophs, or it can be fermented to methane by anaerobic methanogens.

The methylated amines, (mono-, di-, and trimethylamine) occur in

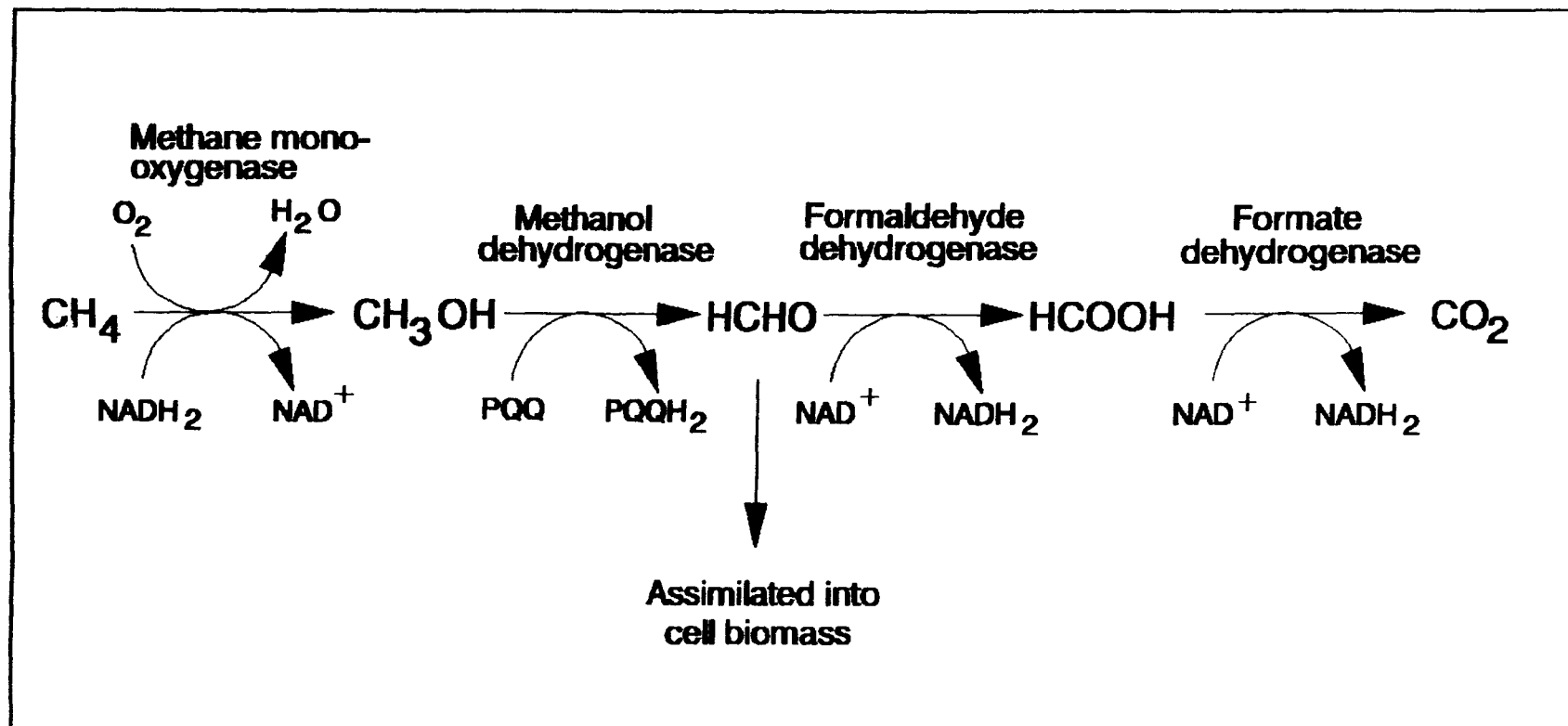
nature as a result of fish decomposition. The methylamines can be utilized by a wide range of methylotrophs (Anthony, 1982).

Other carbon compounds that can be utilized by methylotrophs to provide carbon sources are formaldehyde and formate. Formaldehyde is found in very low concentrations in the environment, because it is very reactive. Formate, in the form of formic acid, is found in the effluent from various industrial processes, where it can serve as an energy source for aerobic methylotrophs or anaerobic methanogens (Large & Bamforth, 1988).

### 1.1.3 Methylotrophs

Methylotrophs can be subdivided into facultative methylotrophs and obligate methylotrophs. Facultative methylotrophs are those bacteria that have the ability to grow on methanol and related  $C_1$  compounds as well as a variety of organic multi-carbon sources. Obligate methylotrophs however can only obtain their carbon and energy from  $C_1$  compounds. Obligate methylotrophs can be further divided into those bacteria that can oxidize methane, also called methanotrophs, and those that cannot.

The bacterial oxidation of methane and methanol is illustrated in fig 1.2. Methane is oxidized to methanol by the enzyme methane monooxygenase. Methanol is oxidized to formaldehyde by methanol dehydrogenase. Formaldehyde is either metabolized further via formate to carbon dioxide to provide reducing power for methane monooxygenase activity, or is assimilated into cell carbon. The carbon dioxide produced by methane oxidation is generally released into the atmosphere where it becomes available as the carbon source for autotrophic



*Fig. 1.2: Bacterial methane oxidation pathway*

and photosynthetic bacteria and plants. Some methylotrophs, however, have the ability to fix carbon from carbon dioxide. This is described in detail in section 1.5.1. The assimilation of formaldehyde into cell carbon in methylotrophs proceeds via one of two pathways, the serine pathway or the ribulose monophosphate pathway. The enzymes involved with the bacterial oxidation of methane and methanol and the two carbon assimilation pathways found in methanotrophs are described in sections 1.5 and 1.6

Methylotrophs are found in a broad spectrum of bacterial groups. There are a number of taxonomic, morphological and biochemical differences between the various groups of methylotrophs, and these are summarized below. The characteristics of methanotrophs are described separately in section 1.3.

Obligate methylotrophs that cannot oxidize methane are generally Gram negative rod-shaped bacteria. The following descriptions of obligate methylotrophs are taken from Anthony, 1982. They are non-pigmented, non-sporing and are motile with a single polar flagellum. The majority are catalase and oxidase positive. They all grow well on methanol, some grow on methylamine and a few can also grow on formaldehyde as a sole carbon source. A small number can also utilize a restricted range of multicarbon compounds such as glucose. These bacteria are known as restricted facultative methylotrophs. All obligate methylotrophs assimilate formaldehyde by the ribulose monophosphate pathway. Ammonia and nitrate are the predominant nitrogen sources, although some methylotrophs can also utilize urea as a nitrogen source. Obligate non-methane utilizing methylotrophs do not possess the ability to fix nitrogen. Taxonomic studies on obligate

methylotrophs (Byrom, 1981; Jenkins & Jones, 1987; Green, 1991) indicate that they can be clustered into two groups: obligate methylotrophs and restricted methylotrophs. This was based on the carbon sources utilised, fatty acid composition, lipid composition and DNA mol% G+C ratio (Jenkins & Jones, 1987: Jenkins et al, 1987). Further classification studies were performed on obligate and restricted methylotrophs by other workers, including polyacrylamide gel electrophoretic studies of whole cell proteins (Byrom, 1981), and the electrophoretic patterns of various enzymes and DNA-DNA homology studies (Urakami & Komagata, 1981; Urakami et al, 1985). The two groups are closely related to each other and it has been proposed that the obligate methylotrophs be placed into the genus Methylobacillus and the restricted methylotrophs assigned to the genus Methylophilus (Jenkins & Jones, 1987: Jenkins et al, 1987). Bacteria assigned to the genus Methylobacillus are short Gram negative rods and are motile with a single polar flagellum or are non-motile. They cannot utilize glucose or fructose as carbon sources but can grow on monomethylamine and methanol. A few can grow on di- or trimethylamine. DNA base composition ranges from between 50 to 56 mol% G+C. Other obligate methylotroph species include Pseudomonas and non-methane utilizing Methylomonas species. Most restricted facultative methylotrophs utilize glucose and methanol as sole carbon sources and methylated amines, formate and fructose are also utilized. DNA base composition ranges from between 50 to 53 mol% G+C.

The genus Hyphomicrobium (Harder et al, 1973; Harder & Attwood, 1978) contain strains that can utilize methanol, methylated amines and formate. Many are restricted facultative methylotrophs, and isolates

described by Attwood & Harder (1972) appear to be obligate methylotrophs. Hyphomicrobium strains have been isolated which show anaerobic growth on methanol. Nitrate or nitrite were both used as the terminal electron acceptor, replacing oxygen (Sperl & Hoare, 1971).

Facultative methylotrophs are a diverse group of heterotrophic organisms related only by their ability to oxidize methanol. Among the facultative methylotrophs are several distinct groups of bacteria. These include the pink pigmented facultative methylotrophs named the Methylobacterium genus (Green & Bousfield, 1983). These have been studied in detail (Green & Bousfield, 1981; Hood et al, 1987; Hood et al, 1988) and appear to fall into four defined groups within the Methylobacterium genus. The pink pigmented facultative methylotrophs (PPFMs) all assimilate formaldehyde into the cell by the serine pathway, as do Type II methanotrophs (see section 1.5.1) and Hyphomicrobium species. A full description of Methylobacterium can be found in Green, 1991. PPFMs grow slowly on nutrient agar and on methanol media. The DNA mol% G+C values range between 68.4 and 72.4 (Hood et al, 1987). Other facultative methylotrophs include some atypical Pseudomonas species and related bacteria, which also demonstrate the ability to utilize C<sub>1</sub> compounds (Yamada et al, 1977; Green, 1991). Pseudomonas species can grow on methylated amines, but not on methanol. The genus Blastobacter contains members that can utilize either methanol or methylated amines or both (Green, 1991). Pseudomonas and Blastobacter methylotrophic species show budding, or polar growth. Some Hyphomicrobium species have been described as facultative methylotrophs in addition to the restricted facultative methylotrophs described above (Jenkins & Jones, 1987). This work also

described facultative methylotrophic Xanthobacter species. There are several lesser groups of Gram negative heterotrophic bacteria that have the ability to utilize C<sub>1</sub> compounds and these are reviewed by Green, 1991.

In addition to the Gram negative bacteria described above, there are also several species of Gram positive bacteria that utilize C<sub>1</sub> compounds. These include Arthrobacter strains (Colby & Zatman, 1973; Kuono & Ozaki, 1975; Levering et al, 1981), and Bacillus species (Colby & Zatman, 1975). Characterization of thermotolerant methylotrophic Bacillus strains isolated from a range of environments (Dijkhuizen et al, 1988) showed that the methanol dehydrogenase present in these Gram positive methylotrophs was different to that found in Gram negative bacteria. Whereas the Gram negative bacteria have a dye-linked methanol dehydrogenase, that of Bacillus strains contain an NAD-dependent enzyme. The methanol dehydrogenases are described in detail in section 1.6.2. Methylotrophic Bacillus strains were found to assimilate carbon into the cell by the ribulose monophosphate pathway. They demonstrated growth on a variety of carbon sources, including methanol, glucose, maltose, mannitol, pyruvate and nutrient broth.

Some methanogens, methane-producing bacteria, are also methylotrophic. Although most methanogenic bacteria ferment acetate and carbon dioxide to methane, some ferment methanol and methylamines (Large & Bamforth, 1988). The rate of methane production by Methanosarcina barkeri from fermentation of methanol is higher than that obtained by fermentation of acetate (Hutten et al, 1980). Methanogens are archaebacteria and are therefore phylogenetically

distinct from methylotrophs.

The final group of microorganisms that utilize  $C_1$  compounds is the methylotrophic yeasts. These organisms mainly utilize methanol, although there are a small number of yeasts that are able to grow on methane and methylamine. Most methylotrophic yeasts are from three genera; Candida, Hansenula and Pichia. Methanol oxidation by different genera proceeds by similar pathways. Utilization of methanol by yeasts is restricted to a few species. These are generally found in habitats rich in pectin and lignin, which contain methoxy groups, such as leaves, soil and wood. Many more yeast species possess the ability to oxidize methanol than can utilize it (Large & Bamforth, 1988). The oxidation of methanol proceeds via an alcohol oxidase situated in peroxisomes (Veenhuis et al, 1983). Methanol is oxidized to formaldehyde and hydrogen peroxide ( $H_2O_2$ ).  $H_2O_2$  is converted by catalase to water and oxygen and the formaldehyde is either assimilated into the cell or further metabolized to carbon dioxide. It is converted into cell carbon by a novel carbon assimilation pathway. This pathway is described in detail by Large & Bamforth (1988). Methane-utilizing yeast appear to be less common. The isolation and characterization of a methane-utilizing yeast was first described by Wolf & Hanson (1979; Wolf et al, 1980). The yeasts isolated showed growth on a variety of complex carbon substrates, including glucose, sucrose, raffinose, glycerol and succinate. Growth was also observed on methylamine, trimethylamine, higher hydrocarbons and higher alcohols (ethanol and propanol). No growth was observed on methanol, formaldehyde or formate. Radioisotope analysis showed conversion of methane to carbon dioxide, indicating that methane does not contribute to cell carbon.



Very little has subsequently appeared in the literature on this type of organism.

## 1.2 Occurrence and isolation of methanotrophs

Methanotrophs occur in a wide range of habitats where methane and oxygen are present. Early workers isolated methanotrophs from environments contaminated with hydrocarbons for example Methylococcus capsulatus (Texas) was isolated from sewage sludge (Foster & Davis, 1966) and Pseudomonas methanitrificans was isolated from oil-field soil. Whittenbury and colleagues (1970a; 1970b; Davies & Whittenbury, 1970) isolated more than one hundred methanotrophs from a variety of natural environments including mud and water from ponds, rivers, streams, ditches and soil samples from the U.K., Russia, France, Germany, North and South America, East and North Africa and Egypt. The success of this work indicates that methanotrophs are relatively ubiquitous. Methanotrophs have been isolated from canals (Hazeu & Steennis, 1970) and from oceans (Sieburth et al, 1987; Lidstrom, 1988). Thermophilic methanotrophs have also been isolated (Shen et al, 1982). Methane oxidation rates can offer an indication of methanotrophic activity, and oxidation rates have been measured in many areas including deep and surface ocean water (Scranton & Brewer, 1978; Sansone & Martens, 1978; Ward et al, 1987), lakes (Lidstrom & Somers, 1984; Abramochkina et al, 1987), landfill cover soil (Whalen et al, 1990) and temperate forest soils (Steudler et al, 1989). Fluorescent antibody detection has shown the presence of Methylomonas methanica and Methylosinus trichosporium in Cleveland Harbour, Ohio (Reed & Dugan, 1978). These methanotrophs were found to be most abundant just

above the sediment. Methanotrophs are also abundant in the surface layers of lake sediments and high numbers are found in the thermocline of meromictic lakes (Higgins et al, 1981).

Methanotrophs are also found in the gills of bivalves (Cary et al, 1988; Jannasch & Taylor, 1984; Childress et al, 1986). The bivalves dependence on symbiotic bacteria for their carbon supply appears to be a widespread phenomenon (Cavanaugh et al, 1987). Bacteria from the bivalve gills typically oxidise reduced sulphur compounds from their environment and use the energy released to fix carbon dioxide from the surrounding sea water. There have been several reports of symbionts obtaining energy and carbon from methane for mussels isolated from hydrocarbon seepage sites (Childress et al, 1986; Cavanaugh et al, 1987; Cary et al, 1988). Uptake of radiolabelled methane and stable carbon isotope ratios confirm that methane breakdown provides the bivalves with at least some of their nutritional requirements. The growth of mussels was found to be supported by methane alone (Cary et al, 1988), indicating that methanotrophic activity of the bacteria could provide sufficient carbon to promote growth of the mussel.

Enrichment of methanotrophs has been attempted in a number of ways. Bacteria from soil can be enriched by adding the soil directly to a mineral salts medium and incubating the culture in a methane atmosphere until turbidity is observed (Foster & Davis, 1966; Whittenbury et al, 1970a). Methanotrophs can be enriched from water from a variety of sources by adding an inoculum of the water to a mineral salts medium and incubating the culture as above (Whittenbury et al, 1970). Sieburth et al (1987) enriched for methanotrophs from the marine environment by incubating undiluted seawater in the

presence of methane. Formaldehyde was also added to the enrichments and suprisingly, since formaldehyde is generally toxic to many organisms, was reported to enhance the growth of methanotrophs. Thermophilic methanotrophs can be isolated by incubation of enrichment cultures at high temperatures (Shen et al, 1982). Isolation of pure cultures has generally been performed by serial dilution of enrichment cultures onto media solidified with agar. After incubation in a methane atmosphere, individual colonies can be selected (Whittenbury & Dalton, 1981).

### 1.3 Classification of methanotrophs

The classification of methylotrophic bacteria has recently been attempted by Green (1991), and this work provides a useful review of the different taxonomic groups of methylotrophs.

Before 1970, there had been few reports of the isolation of methanotrophs described in the literature. The first report of an isolated methanotroph, Bacillus methanicus, was described by Sohngen (1906). A similar organism was isolated by Dworkin and Foster (1956), and named Pseudomonas methanica. There were only four other methanotrophic strains isolated before 1970: a marine methanotroph (Hutton & Zobell, 1949); Pseudomonas methanitrificans (Davis et al, 1964), Methanomonas methanooxidans (Brown et al, 1964) and Methylococcus capsulatus (Texas) (Foster & Davis, 1966). In 1970, Whittenbury et al (1970a; 1970b; Davies & Whittenbury, 1970) isolated and described more than 100 strains of methanotrophs. Much of this early classification forms the basis of methanotroph characterization today.

Methanotrophs are a diverse group of organisms comprising several

genera. Their morphology is varied, ranging from rods to cocci, coccobacilli and pear-shaped organisms. Rods can be curved or vibroid, slender or stout. All methanotrophs are catalase and oxidase positive. Most methanotrophs form 'resting stages'. Whittenbury et al (1970b) described three varieties of resting stage; an exospore and two types of cyst. Exospores are formed by two methanotroph strains, Methylosinus trichosporium and Methylosinus sporium. Exospores are formed by budding and are heat and desiccation resistant. Cysts are formed by many methanotrophs which do not form exospores and can occur in two distinct varieties. Lipid cysts are formed by only one organism, Methylocystis parvus and, unlike exospores, are not heat resistant. They are, however, resistant to desiccation. The cysts are formed by the inclusion of lipid bodies. Many other methanotroph strains appear to form an 'Azotobacter-like' cyst, including all members of the Methylobacter genus. An immature form of this type of cyst is also seen among Methylomonas and Methylococcus species. 'Azotobacter-like' cysts are desiccation resistant but are not heat resistant.

All methanotrophs thus far studied have complex arrangements of internal membranes. The position of these membrane structures within the cell contributes to the grouping of methanotrophs into Type I or Type II organisms. Type I methanotrophs have bundles of paired membranes distributed throughout the cell whereas Type II organisms have a system of paired peripheral membranes (Davies & Whittenbury, 1970). These membranes are unique to methane-utilizing methylotrophs indicating their possible involvement in the oxidation of methane to methanol (Anthony, 1982).

A second aid to grouping methanotrophs into Type I or Type II organisms can be found by investigating the carbon assimilation enzyme pathways. Methanotrophs assimilate carbon from formaldehyde (formed by conversion of methane to formaldehyde via methanol, see fig 1.2) by one of two pathways. The ribulose monophosphate pathway is used to assimilate carbon by Type I methanotrophs whereas the serine pathway is used by Type II organisms. These pathways are described in detail in section 1.5.1. Methylococcus capsulatus (Bath) does not appear to belong to either Type I or Type II. It possesses internal bundles of membranes and assimilates carbon by the ribulose monophosphate pathway, like the Type I methanotrophs, but appears to have properties in common with the Type II methanotrophs. For example Methylococcus capsulatus (Bath) and Type II methanotrophs have a DNA mol% G+C of approximately 62.5%, compared to that of Type I methanotrophs, which have a DNA mol% G+C of 50-54. Methylococcus capsulatus (Bath) is the only methanotroph so far described which can fix CO<sub>2</sub> via ribulose biphosphate carboxylase (Taylor, 1977). This, together with several other unique features, place Methylococcus capsulatus (Bath) into a third group, Type X.

Methanotrophs can utilize several substrates as nitrogen sources. Methanotrophs isolated by Whittenbury et al (1970a) all use ammonium chloride as a nitrogen source. It was reported, however, that at concentrations of ammonium chloride above 0.05% (w/v), some inhibition of growth rates was observed. This could indicate competitive inhibition of methane monooxygenase by ammonia. Nitrite and nitrate are also used as nitrogen sources by the majority of Whittenbury's isolates. Other nitrogen sources used by methanotrophs include urea,

casamino acids and yeast extract (Whittenbury et al, 1970a) and asparagine, cystine, peptone and tryptone (Green, 1991). The assimilation and incorporation of amino acids by Methylococcus capsulatus (Texas) have been investigated by Eccleston & Kelly (1972a; 1972b) where the incorporation of radiolabelled amino acids (aspartate, glutamate, homoserine, lysine and threonine) was followed. Although this work demonstrated amino acid uptake, the amino acids were not tested as sole nitrogen sources. The only complete study of amino acids as sole nitrogen sources for methanotrophs (Murrell, 1981) showed that generally Type II methanotrophs could utilize a broader spectrum of amino acids as sole nitrogen sources than Type I organisms. Several amino acids (arginine, cysteine, glycine, histidine, lysine and tryptophan) could not be utilized as sole nitrogen sources by any of the methanotrophs tested.

Nitrogen-fixation by a methanotroph was first described by Davis et al (1964), who isolated nitrogen fixing bacteria capable of methane utilization from soil. This was named Pseudomonas methanitrificans. Whittenbury et al (1970a) observed nitrogenase activity by Methylosinus trichosporium PG. Further work by DeBont & Mulder (1974), Dalton & Whittenbury (1975; 1976), DeBont (1976) and Murrell & Dalton (1983a) has found that nitrogen-fixation occurs in all Type II methanotrophs so far tested and also in Methylococcus capsulatus (Bath). Further details of nitrogen fixation are described in section 1.5.2.

Many methanotrophs are motile. The majority of rod-shaped Type I methanotrophs (the Methylomonas and Methylobacter genera) are motile and have a single polar flagellum (Green, 1991). Type II Methylosinus species are also motile and possess tufts of flagella. Other Type II

organisms and coccoid methanotrophs are non-motile. Most motile Type II methanotrophs form rosettes of bacteria held together at their non-flagellated poles by polysaccharide (Whittenbury et al, 1970a).

A useful aid to classification of bacteria is the DNA base composition, or mol% G+C content. This can be calculated by several methods, including DNA thermal denaturation (Mandel & Marmur, 1968) and caesium chloride density gradient ultracentrifugation (Mandel et al, 1968). The DNA base composition has been determined for a number of methanotrophs (Romanovskaya, 1984; Bowman et al, 1990 among others). The DNA base composition is found to be between 50 to 54% mol G+C for Type I organisms, and between 61 to 63% mol G+C for Type II methanotrophs. This provides a further aid to grouping methanotrophs into Type I or Type II organisms. The Type X methanotroph Methylococcus capsulatus (Bath) has a mol G+C of 62.5%. Recent work by Bowman et al (1991), however, has shown that the distinction between Type I methanotrophs and Type II methanotrophs, in terms of their mol% G+C content, is not as clearly defined as previously thought. For example, Methylobacter strains, typical Type I bacteria in all other characteristics, were found to have mol% G+C values of between 50 and 60%. Whilst it is prudent not to define methanotrophs into either Type I or Type II on the basis of G+C content alone, it is still a useful parameter for confirming results from other characteristics.

There have been several attempts to assign methanotrophs to taxonomic groups. In the early work of Whittenbury et al (1970a; 1970b) and Davies & Whittenbury (1970), initial classification was based on cell morphology, fine structure and type of resting stage formed. The groups are still used today as the basis for methanotroph

taxonomy. An attempt to correlate data for representative organisms from published reports was made by Romanovskaya et al (1978) and full descriptions of the taxonomic groups proposed were presented.

Characterization of methanotrophs has been attempted by comparison of fatty acid composition (Romanovskaya et al, 1980; Guckert et al, 1991) and protein profiles of methanotrophs (Gal'chenko & Nesterov, 1980). There appear to be differences between the fatty acid content of Type I and Type II methanotrophs. (Higgins et al, 1981). Type I methanotrophs possess an esterified fatty acid content of the C<sub>16:0</sub>, C<sub>16:1</sub> type, whereas Type II strains possess esterified fatty acids of C<sub>18:1</sub> type. Classification of methylotrophs based on fatty acid content was performed recently by Guckert et al (1991). Results from this study indicated that classification of methylotrophs based on fatty acid content closely followed that of 16SrRNA sequence analysis (Tsuji et al, 1990, discussed below), thus providing a useful taxonomic tool. Gal'chenko & Andreev (1984) proposed a classification system based on several parameters. Phospholipid composition, fatty acid composition, protein profiles, antigenic characteristics and DNA homology were analysed and a classification scheme devised. The results obtained from these studies indicate that Whittenbury's initial classification (Whittenbury 1970a; 1970b and Davies & Whittenbury, 1970) is still valid. This is also the conclusion of P. Green (1991).

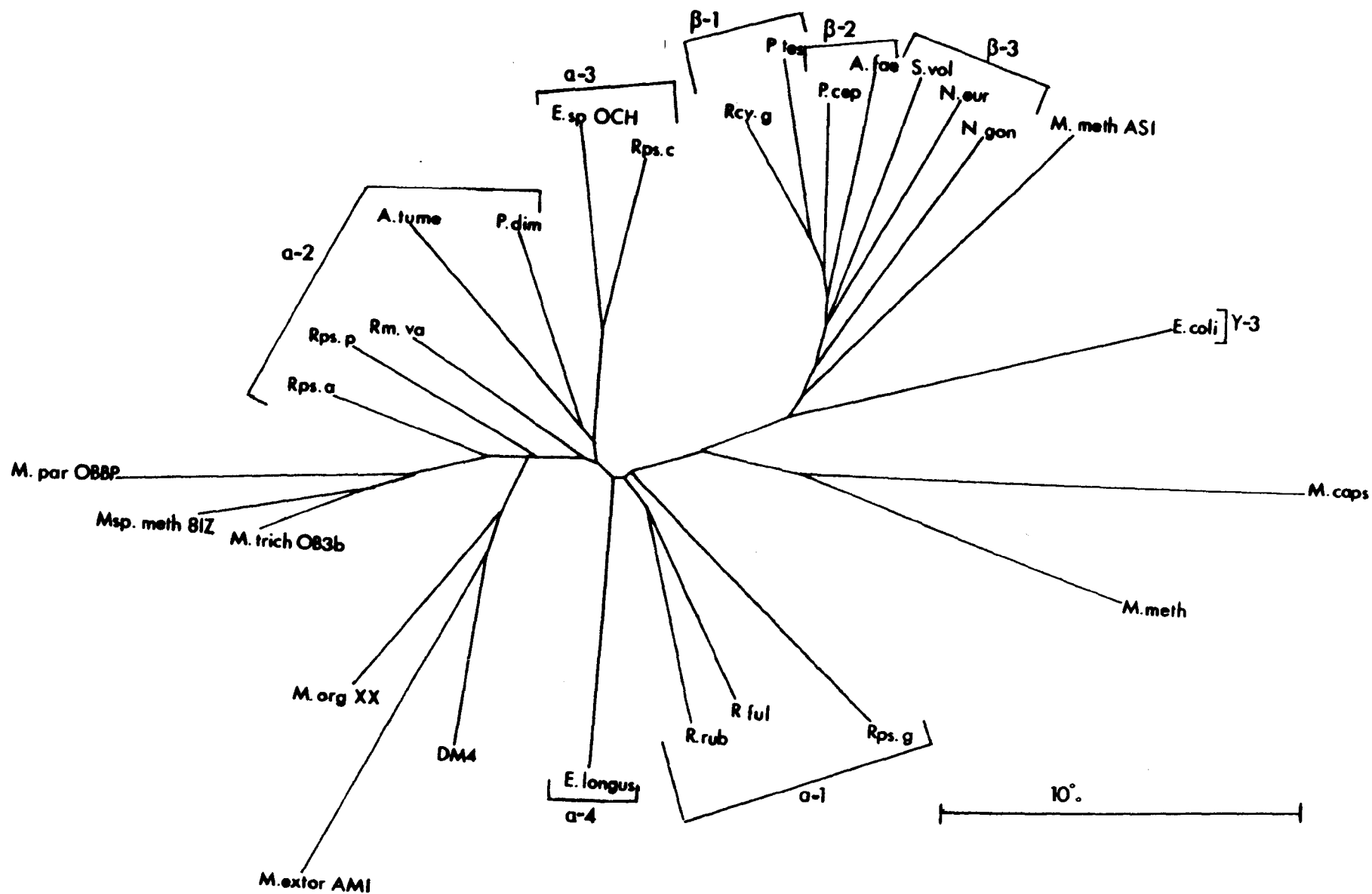
Recently, nucleic acid characterization has been performed on a number of methanotrophs. 16S ribosomal RNA sequences have been analysed (Tsuji et al, 1990) and the phylogenetic relationship of 12 strains of methylotrophs determined. A phylogenetic tree summarizing



the relationship between methylotrophs and other representatives of the Proteobacteria (purple eubacteria) based on 16S rRNA analysis is shown in fig. 1.3. This shows that methanotrophs are not closely related, but are a diverse group scattered throughout the purple eubacteria. However, it is apparent that Type I methylotrophs fall into two groups the  $\beta$ - and the  $\gamma$ -subdivision, and that Type II methylotrophs fall into a third group, the  $\alpha$ -subdivision. The PPfMs and Hyphomicrobium also fall into this group. This is interesting as all of these bacteria assimilate carbon into the cell by the serine pathway, whereas all other methylotrophs utilize the ribulose monophosphate pathway. Genome characteristics have also been analysed (Bowman et al, 1991). Genome size and DNA base composition were studied in 48 methanotroph strains and the similarity between strains has been determined. The relationships determined from this study are shown in fig. 1.4. The classification derived from this analysis broadly follows Whittenbury's early work (Whittenbury 1970a; 1970b and Davies & Whittenbury, 1970). The characteristics for each group are summarised in table 1.3.

#### 1.4 Marine methylotrophs

The first description of a marine methylotroph was that of Hutton & Zobell (1949) who isolated a methane-utilizing bacterium that did not grow on more complex carbon sources. Budd & Spencer (1968; Budd, 1969) described a trimethylamine-utilizing Pseudomonas species. Isolation of methanol-utilizing bacteria in the marine environment was first described by Yamamoto et al, 1978, who isolated sixty-five strains of methanol utilizing bacteria, and classified these into



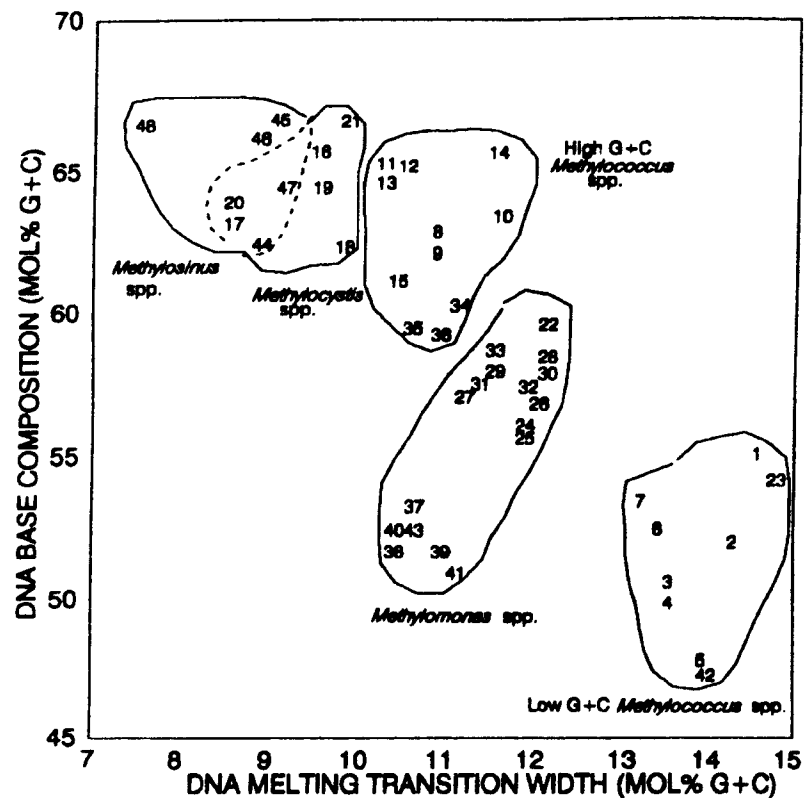


Fig. 1.4a: Similarity map of obligately methanotrophic bacteria based on DNA base composition and nucleotide distribution. From Bowman *et al* 1991.

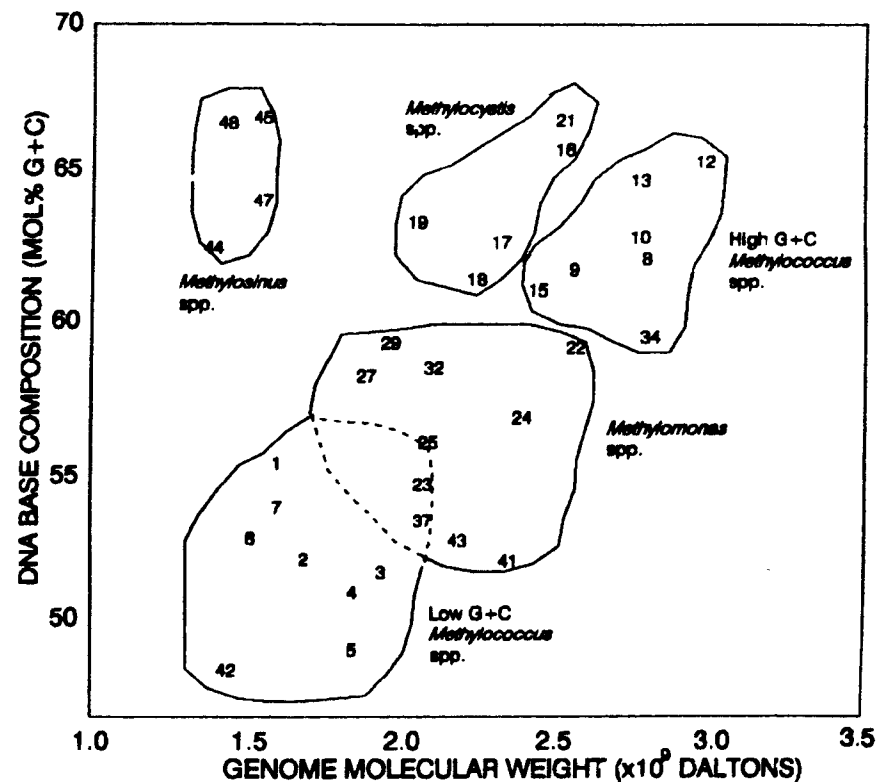


Fig. 1.4b: Similarity map of obligately methanotrophic bacteria based on DNA base compositions and genome molecular weights. See text for further details. From Bowman *et al* 1991.

CHARACTER	TYPE I	TYPE X	TYPE II
Morphology	straight rod	coccus	curved, pear-shaped or straight rod
Motility	+/-	-	+/-
Resting stage	Azotobacter-type cyst	Azotobacter-type cyst	lipid cyst or terminal exospore
Rosette formation	-	-	+ (most strains)
Carbon assimilation	RUMP	RUMP	Serine
CO <sub>2</sub> fixation	-	+	-
Complete TCA	-	-	+
Nitrogenase	-	+	+
Fatty acid carbon chain length	16	16	18
Mol %G+C*	50-54	62.5	61.7-63.1
sMMO	-	+	+ (some strains)
Genera	<i>Methylomonas</i> <i>Methylobacter</i> <i>Methylococcus</i>	<i>Methylococcus</i> <i>capsulatus</i> (Bath)	<i>Methylosinus</i> <i>Methylocystis</i>

Table 1.3: Methanotroph characteristics (adapted from Green, 1991).

\* recent work has shown that the mol% G+C content of methanotrophs may be less rigid than that shown here (see text for further details).

obligate and facultative methylotrophs (Yamamoto et al, 1980). Further characterization of marine obligate methylotroph isolates has been described by Strand & Lidstrom (1984), Urakami & Komagata (1987) and Kimura et al (1990a, 1990b, 1990c). All of the marine methylotrophs described above show sodium chloride dependent growth. The ribulose monophosphate pathway appears to be used for carbon assimilation in all strains tested. No marine methylotrophs that assimilate carbon solely by the serine pathway have been described so far. All strains are described as Gram negative rods with polar flagella. Apart from the requirement for sodium chloride, the marine strains appear to have similar characteristics to those of freshwater methylotroph strains.

The methane-utilizing bacteria isolated from the marine environment by Hutton & Zobell (1949) were not well characterized and the isolates were not preserved. Sansone & Martens, 1978, reported isolation of methanotrophs from Cape Lookout Bight, North Carolina, but no characterization of the bacteria was reported. Sieburth et al, 1987, published the first characterization studies of a marine methanotroph, Methylomonas pelagica. These bacteria were obligate type I methanotrophs and were Gram negative short motile rods with a single polar flagellum. Sodium chloride was required for growth. The isolates could utilize both methanol and methane. Carbon was assimilated by the ribulose monophosphate pathway. Lidstrom, 1988, described methanotrophs isolated from a sewage outfall region near Los Angeles. These bacteria also appeared to be Type I methanotrophs, but contained both serine pathway and ribulose monophosphate pathway enzymes. Other characteristics appeared similar to the bacteria isolated by Sieburth.

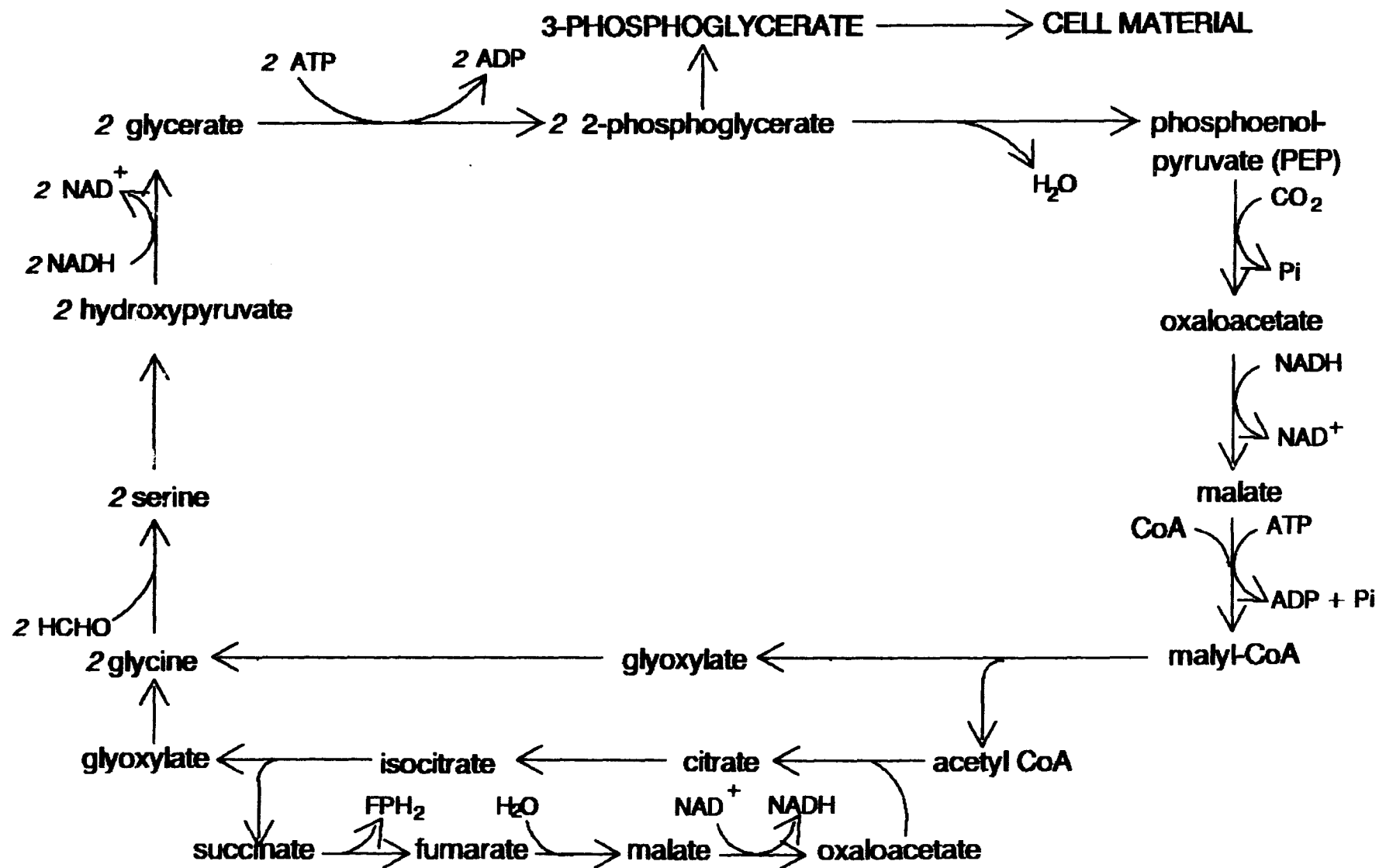
The bacteria isolated by Lidstom, however, had a much lower concentration of sodium chloride required for optimum growth.

## 1.5 Physiology and biochemistry of methanotrophs

### 1.5.1 Carbon assimilation pathway enzymes

Methanotrophs oxidize methane to methanol, which is further oxidized to formaldehyde (fig 1.2). Formaldehyde is then either dissimilated to carbon dioxide via formate to provide reducing power for methane monooxygenase activity, or is assimilated to cell carbon. In methanotrophs, the assimilation of formaldehyde can proceed by one of two pathways, the serine pathway or the ribulose monophosphate pathway. These two pathways are illustrated in figs 1.5 and 1.6. Methylococcus capsulatus (Bath) is the only methanotroph so far described that contains ribulose biphosphate carboxylase. This will be briefly described below.

Type I methanotrophs and Methylococcus capsulatus assimilate cell carbon by the ribulose monophosphate pathway (RuMP). This pathway was first proposed by Kemp & Quayle (1967) from evidence obtained by incubating Methylomonas methanica with  $^{14}\text{C}$ -labelled methanol and formaldehyde. The following description of the pathway is taken from Large & Bamforth (1988). The cycle can be described in three stages. The first stage is a fixation step and is a condensation between three  $\text{C}_1$  units (formaldehyde) and three  $\text{C}_5$  sugar phosphates (ribulose-5-phosphate) to give three  $\text{C}_6$  sugars (hexulose-6-phosphate) This is followed by an isomerization of hexulose-6-phosphate to fructose-6-phosphate. The second stage of the pathway involves a cleavage of the fructose-6-phosphate via 2-keto 3-deoxy 6-phosphogluconate (KDPG) to



**Fig. 1.5: Serine pathway of carbon assimilation (after Anthony, 1982)**

**Fig. 1.6: Ribulose monophosphate pathway of carbon assimilation (after Anthony, 1982)**



pyruvate and glyceraldehyde phosphate. The pyruvate is used for biosynthesis whilst the remaining fifteen carbon atoms undergo the third stage of rearrangement to form the original three  $C_5$  molecules and the cycle continues. There is an alternative cleavage step found in facultative methylotrophs in which dihydroxyacetone phosphate is produced in place of pyruvate.

Type II methanotrophs assimilate carbon by the serine pathway. This pathway has amino acids and carboxylic acids as the intermediates, instead of the carbohydrates found in the ribulose monophosphate pathway. The following description is taken from Anthony (1982). Two molecules of formaldehyde are combined with two molecules of glycine to give (through serine, hydroxypyruvate and glycerate) two molecules of 2-phosphoglycerate. One molecule is assimilated into cell carbon through 3-phosphoglycerate, whilst the other molecule is converted to phosphoenolpyruvate (PEP). PEP is catalysed to malyl-coenzyme A which is then cleaved to give glyoxylate and acetyl-coenzyme A. Acetyl-coenzyme A then passes through a glyoxylate cycle to produce glyoxylate, then glycine and the cycle begins again.

The enzymes involved in the assimilation of carbon in methanotrophs have been assayed in a number of bacteria. By measuring the activity of key enzymes in both pathways, the major pathway of assimilation can be determined. The activity of key enzymes in the carbon assimilation pathways now forms part of the characterization of new species.

The presence of the ribulose monophosphate pathway can be determined by assaying for activity of hexulose phosphate synthase, which catalyses the first step in the pathway, the condensation of

formaldehyde with ribulose-5-phosphate. This was first measured by radioactive formaldehyde condensation (Lawrence et al, 1970). Hexulose phosphate synthase can also be measured spectrophotometrically by following the rate of ribulose-5-phosphate and formaldehyde dependent formation of hexulose phosphate. This was described by Quayle (1982). Ribulose monophosphate pathway enzymes have been assayed in many methanotrophs including: Methylococcus capsulatus by Lawrence et al (1970); Methylomonas agile, Methylomonas rosaceus, Methylomonas methanica, Methylobacter capsulatus & Methylococcus minimus by Lawrence & Quayle (1970); and Methylococcus capsulatus & Methylomonas methanica Strom et al (1974). The ribulose monophosphate pathway has also been demonstrated in obligate non-methane utilizing methylotrophs and in a few facultative methylotrophs, for a review, see Anthony, 1982.

The serine pathway is investigated by measuring the activity of several key enzymes. Serine transhydroxymethylase, the enzyme that catalyses the formation of serine by condensation of formaldehyde and glycine, also catalyses the biosynthesis of glycine from serine during growth on multi-carbon compounds and is probably present in most bacteria. Therefore it cannot be used as an indicator enzyme for the serine pathway. The key enzyme used to determine the presence of the serine pathway in methanotrophs is hydroxypyruvate reductase. This enzyme catalyses the reduction of hydroxypyruvate to glycerate. Hydroxypyruvate reductase can be assayed colorimetrically by following the hydroxypyruvate-dependent oxidation of NADH to NAD<sup>+</sup> (Large & Quayle, 1963). Serine-glyoxylate aminotransferase activity is a second key enzyme that is commonly assayed to determine serine pathway

activity and can be assayed colorimetrically by following the formation of glyoxylate dependent formation of hydroxypyruvate from serine (Harder & Quayle, 1971). Methanotrophs shown to possess the serine pathway include: Methanomonas methanooxidans by Lawrence et al (1970); Methylovibrio soehngenii by Hazeu & Steenis (1970) and Methylosinus trichosporium, Methylosinus sporium & Methylocystis parvus by Lawrence & Quayle (1970). The serine pathway has not been found in any of the obligate non-methane utilizing methylotrophs, but is commonly seen in facultative methylotrophs.

With the exception of Methylococcus capsulatus, methanotrophs do not possess the ability to assimilate carbon dioxide into the cell via ribulose biphosphate carboxylase. The pathway found in Methylococcus capsulatus (Bath) appears to be identical to that found in autotrophs (Taylor et al, 1980). In the ribulose biphosphate pathway (or Calvin cycle) of carbon dioxide assimilation (fig 1.7), three molecules of carbon dioxide are combined with three molecules of ribulose 1,5-bisphosphate to give six molecules of phosphoglycerate. These are further phosphorylated and then reduced to six glyceraldehyde phosphate molecules. One of these is then assimilated into the cell, whilst the remaining five molecules rearrange to give the initial three ribulose 1,5-bisphosphate molecules (Anthony, 1982). Taylor (1977) demonstrated the presence of ribulose 1,5-bisphosphate carboxylase, the enzyme responsible for combining the carbon dioxide molecules to ribulose 1,5-bisphosphate, in Methylococcus capsulatus (Bath). This enzyme was subsequently purified from M. capsulatus (Bath) (Taylor et al, 1980) and further investigation into the activity (Taylor et al, 1981; Stanley & Dalton, 1982) indicated that

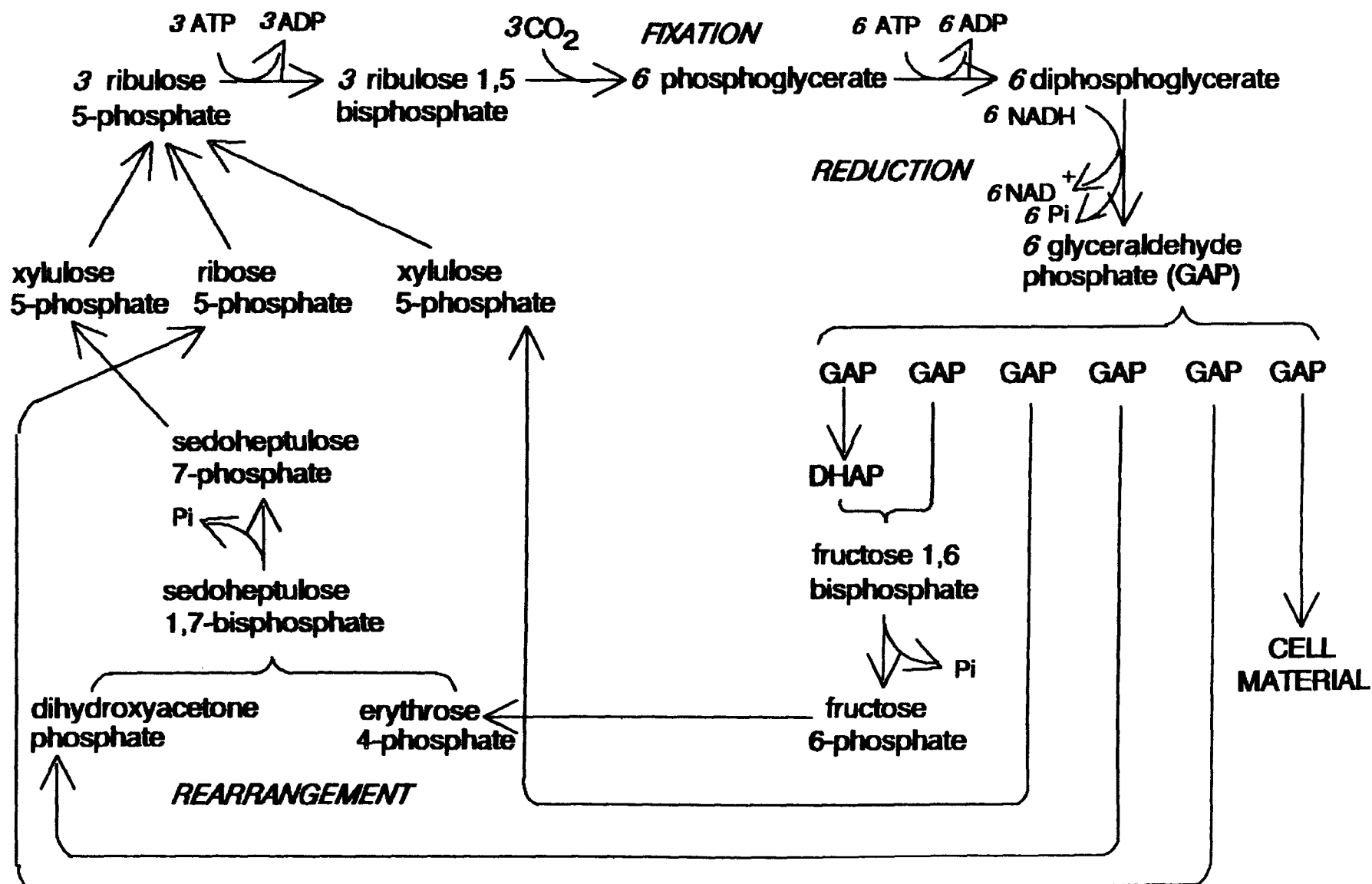


Fig. 1.7: Calvin cycle of carbon fixation - sedoheptulose biphosphate variant.

whilst Methylococcus capsulatus (Bath) appears to have all the enzymes for carbon dioxide assimilation, it was unable to grow autotrophically on carbon dioxide alone. It was estimated that no more than 2.5% cell carbon originated from carbon dioxide (Taylor et al, 1981) when methane was present.

In addition to the carbon assimilation pathways described above, methanotrophs have metabolic blocks in energetic and biosynthetic pathways. This may explain why methanotrophs are obligate methane and methanol utilizers and cannot grow on more complex carbon sources. This was studied by Shishkina & Trotsenko (1979). The main conclusions from this work was that in Type I methanotrophs, complex carbon compounds cannot be fully oxidized because they lack 2-oxoglutarate-dehydrogenase, a key enzyme in the tricarboxylic (TCA) cycle. In addition, these bacteria do not possess isocitrate lyase and malate synthase, which are essential for the glyoxylate shunt involved with biosynthetic pathways for complex carbon sources. Type II methanotrophs appeared to have all the enzymes necessary for a complete TCA cycle. However, isocitrate lyase and malate synthase are again absent. Type II methanotrophs cannot synthesize PEP from pyruvate because they lack the essential enzymes for this step. In all methanotrophs, pyruvate kinase is absent, thus PEP cannot be converted to pyruvate. Shishkina & Trotsenko (1979) concluded that the inability of methanotrophs to grow on any carbon compound other than methane or methanol was due to a number of enzymic lesions, rather than just one step.

### 1.5.2 Nitrogen assimilation pathway enzymes

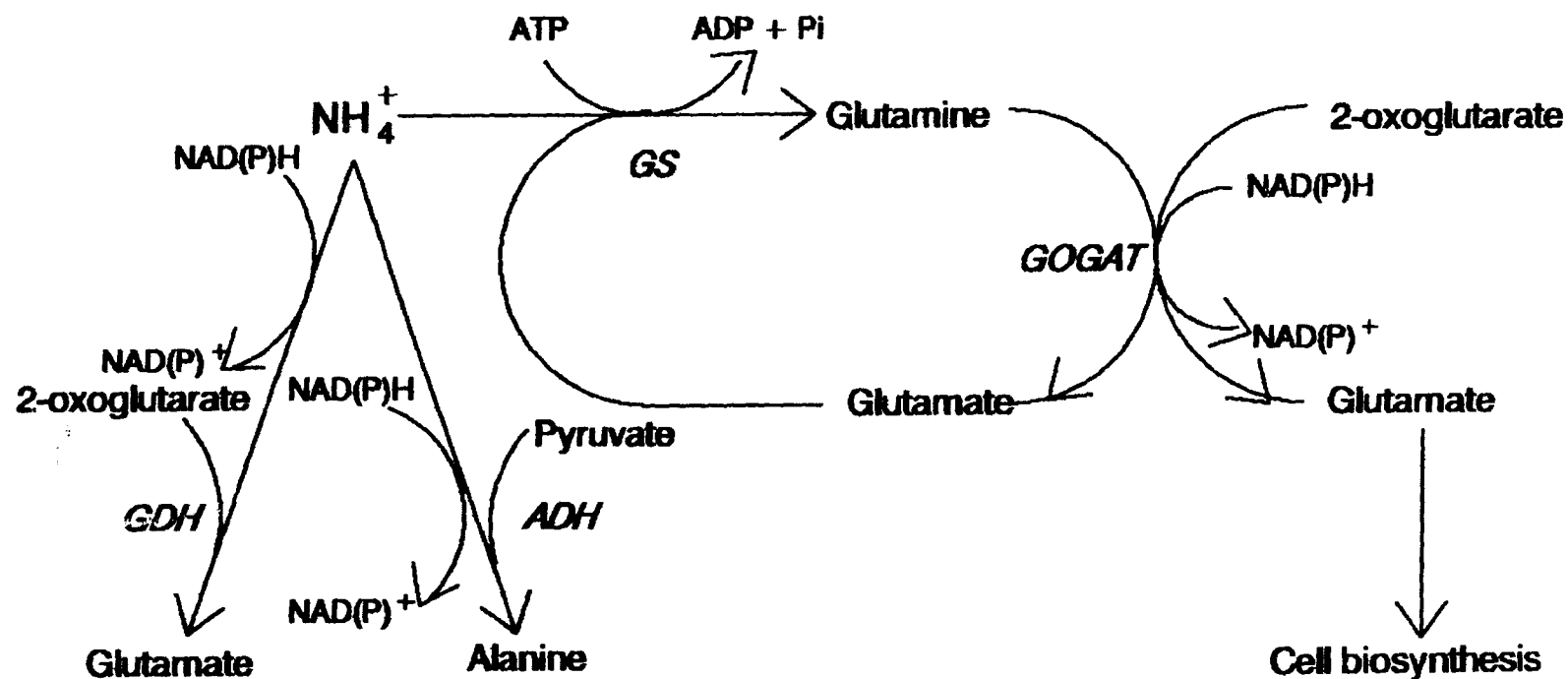
Nitrogen metabolism in bacteria involves the assimilation of ammonia into the bacterial cell. Several inorganic nitrogen compounds such as dinitrogen ( $N_2$ ), nitrite, nitrate or ammonia may be taken into the cell as nitrogen sources as well as more complex organic compounds such as amino acids, but all of these must be converted to ammonia before assimilation. There are several pathways for ammonia assimilation in bacteria which usually proceed via glutamine and glutamate. When the extracellular concentration of ammonia is high, ammonia is assimilated by glutamate dehydrogenase (GDH) and alanine dehydrogenase (ADH) which typically have high values for the  $K_m$  for ammonia. In conditions where the extracellular concentration of ammonia is low, the high  $K_m$  values for ammonia of GDH and ADH indicate that the assimilation of ammonia by these enzymes is inefficient. This led to the discovery of a second pathway of ammonia assimilation by glutamate synthase [Glutamine (amide): 2-oxoglutarate amino-transferase oxidoreductase ( $NADP^+$ ) or GOGAT] (Tempest et al, 1970; Meers et al, 1970). Together with glutamine synthetase (GS), GOGAT was seen to be responsible for the assimilation of ammonia under conditions of extracellular ammonia limitation. The  $K_m$  value of GS for ammonia was found to be typically less than 1 mM (Brown & Herbert, 1977; Kleinschmidt & Kleiner, 1978). Similarly, the  $K_m$  for glutamine of GOGAT in several bacteria was found to be between 0.1 to 2 mM (Meers et al, 1970). These low values indicate that, for most bacteria at least, ammonia is assimilated primarily by GS/GOGAT when the ammonia concentration is low. When extracellular ammonia is found in high concentrations, it is assimilated by GDH or ADH. The pathways of

ammonia assimilation are illustrated in fig. 1.8.

Glutamate dehydrogenase is a reversible enzyme and appears to have a catabolic action in most bacteria, forming ammonia from glutamate and only has an anabolic action in high ammonia concentrations. GDH activity can be either NADH- or NADPH-linked and most bacteria only possess one type of GDH activity. Some bacteria, however, demonstrate both types of activity, as was seen in Hydrogenomonas H16 (Kramer, 1970). The NADPH-linked enzyme was found in cells with high ammonia concentrations, whilst the NADH-linked enzyme was present when ammonia was limiting which indicated that this enzyme had a catabolic function. The NADPH-linked enzyme showed biosynthetic activity. The conclusions from this work was supported by results from the observed activities of NADH- and NADPH-linked GDH in several Pseudomonas species. The NADPH-linked enzyme was expressed in high ammonia concentrations, which also inhibited the GS/GOGAT function, indicating that the NADPH-linked enzyme was acting biosynthetically. An NADH-linked enzyme was ascribed a catabolic function.

Alanine dehydrogenase catalyses the reversible deamination of pyruvate to alanine (see fig. 1.8). Unlike GDH, ADH is present in a limited number of bacteria, for example Rhodopseudomonas capsulata (Johannson & Gest, 1976). The  $K_m$  values for ammonia of ADH is invariably high. A value of 300 mM was found in Bacillus licheniformis (Meers & Pedersen, 1972). Like GDH, ADH appears to only function biosynthetically under high ammonia concentrations and its main function is catabolic.

The assimilation of ammonia by GS/GOGAT has been well documented in many species of bacteria. In ammonia-limiting conditions, GS and



**Fig. 1.8: Alternative pathways of bacterial ammonia assimilation**

**ADH**- Alanine dehydrogenase

**GDH**- Glutamate dehydrogenase

**GS**- Glutamine synthetase

**GOGAT**- Glutamate synthase



GOGAT have high activities. Bacteria which lack GDH, for example Erwinia carotovora (Meers et al, 1970) and Rhodopseudomonas capsulata (Johansson & Gest, 1976) show constitutive expression of GOGAT. Purification of GOGAT from several bacteria [e.g. Escherichia coli (Miller & Stadtman, 1972) and Klebsiella aerogenes (Trotta et al, 1974)] has shown it to be an iron-sulphur flavoprotein and highly specific for L-glutamine, 2-oxoglutarate and either NADH or NADPH.

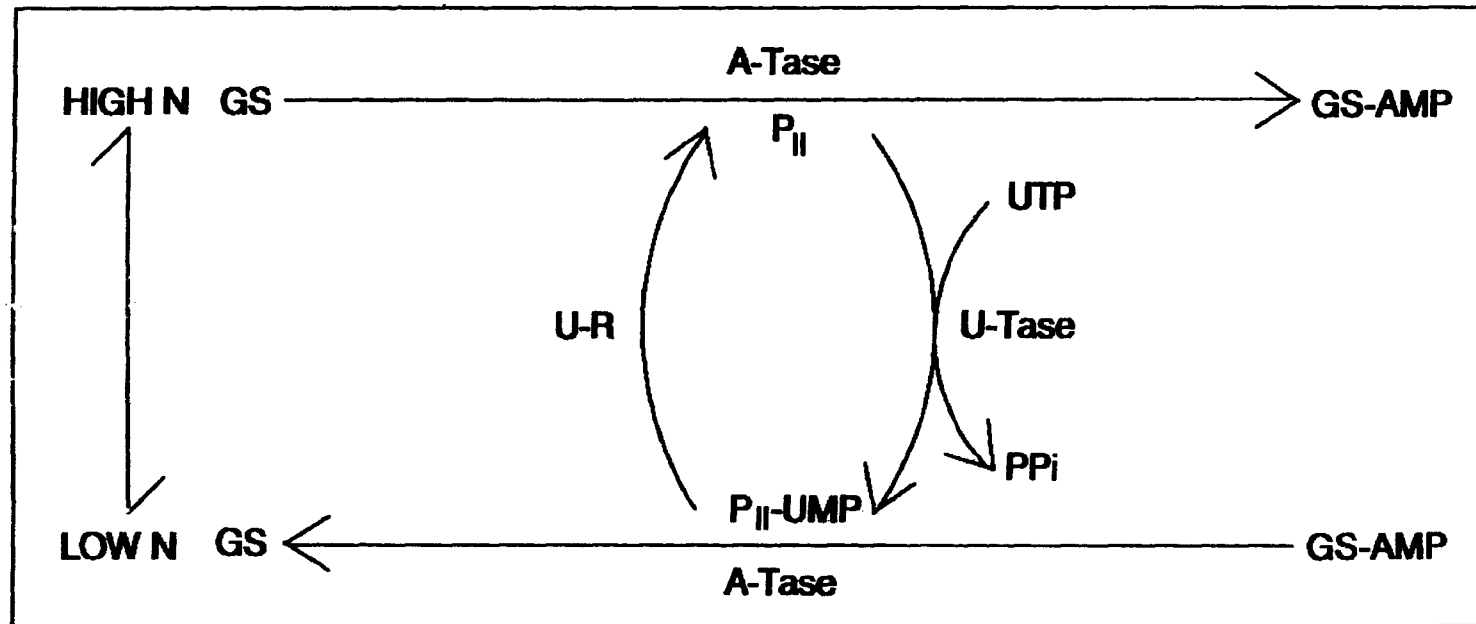
Glutamine synthetase (GS) catalyses the synthesis of glutamine from glutamate and ammonia. Glutamine is essential for the biosynthesis of several amino acids, such as asparagine, histidine and tryptophan. It is also involved in the formation of amino sugars, carbamyl phosphate, purine and pyrimidine nucleotides and NAD. GS has, therefore, an essential role in bacteria and has been studied in detail. The characterization of GS was reviewed by Ginsburg & Stadtman, 1973. It is a dodecameric protein of  $M_r$  600,000 made up of 12 identical subunits of  $M_r$  50,000. In high concentrations of extracellular ammonia, GS rapidly loses its ability to form glutamine. This effect is reversed by removal of excess ammonia. This points to tight controls over the activity of GS. GS activity is controlled by three separate systems:

- adenylation/deadenylation control
- modification of GS by divalent cations
- cumulative feedback inhibition

Adenylation of GS occurs when an adenylyl group attaches to a specific tyrosine residue on each subunit. Adenylation is catalysed by adenylyl transferase in an ATP dependant reaction. Adenylyl transferase is itself controlled by a regulatory protein  $P_{II}$ . Deadenyly-

lation of GS is also controlled by adenylyl transferase and  $P_{II}$ .  $P_{II}$  is found in one of two forms in the cell:  $P_{IID}$  is a modified form in which uridylyl groups are attached and is essential for deadenylylation whereas the unmodified form,  $P_{IIA}$ , is necessary for adenylylation. The form of  $P_{II}$  therefore determines whether GS is adenylylated or deadenylylated. Uridylyl groups are attached to the  $P_{II}$  protein by the action of the enzyme uridylyl transferase (UTase) and are removed by another function of the enzyme (UR). Adenylyl transferase and UTase are controlled by intracellular levels of glutamine and 2-oxoglutarate. Under high levels of glutamine the adenylyl transferase  $P_{IIA}$  complex is formed, stimulating adenylylation of GS and halting the biosynthesis of more glutamine. When the levels of 2-oxoglutarate are high, the reverse occurs. The UTase enzyme is stimulated, the  $P_{II}$  protein becomes uridylated which causes deadenylylation of GS and therefore formation of glutamine. The final control of this regulation is ammonia, which determines the levels of glutamine and 2-oxoglutarate. The mechanism of GS control is illustrated in fig. 1.9.

GS can be modified by fluctuation in the concentration of divalent cations. GS requires the presence of  $Mn^{2+}$  or  $Mg^{2+}$  and removal of these cations results in inactivation of the enzyme. Removal of  $Mn^{2+}$  causes a relaxation of the GS protein which leads to greater susceptibility for dissociation of the subunits. Addition of  $Mn^{2+}$  and  $Mg^{2+}$  completely reverses this effect and restores GS activity. The metal ion specificity of GS changes with the adenylation of GS. The deadenylylated form of GS requires  $Mg^{2+}$  for activity whereas the adenylylated form requires  $Mn^{2+}$  but not  $Mg^{2+}$  for a low level of biosynthetic



**Fig. 1.9: Regulation control of glutamine synthetase by adenylation  
(after Merrick, 1988)**

- GS** - deadenylylated glutamine synthetase
- GS-AMP** - adenylylated glutamine synthetase (inactive)
- A-Tase** - adenylyltransferase
- U-Tase** - uridylyltransferase
- U-R** - uridylyl-removing enzyme

activity.

Cumulative feedback inhibition may also play a role in the control of GS activity. Regulation through feedback is controlled by the end products of glutamine metabolism (histidine, tryptophan, AMP, CTP, carbamyl phosphate etc.). This control been demonstrated in many bacteria, for example; Rhodopseudomonas capsulata (Johansson & Gest, 1976).

Ammonia assimilation in methanotrophs was first studied by Shishkina & Trotsenko (1979) who observed that in cell-free extracts Type I methanotrophs did not show any activity of GOGAT. ADH, GDH and GS all demonstrated measurable activity and they concluded that this was how ammonia was assimilated into the cells of Type I methanotrophs. With Type II methanotrophs, however, it was proposed by the same workers that ammonia was assimilated exclusively by GS/GOGAT, as there was no evidence for amino acid dehydrogenase activities. For Methylococcus capsulatus (Foster & Davis strain) they proposed that, in addition to ADH, GS/GOGAT was used to assimilate ammonia in cells during growth on N<sub>2</sub>. This is suprising as most organisms which possess ADH, the activities are highest in cell-free extracts from ammonium grown cells.

There was little further examination of ammonia assimilation in methanotrophs until 1983, when Murrell & Dalton (1983b) investigated the ammonia assimilation in a Type I methanotroph (Methylomonas methanica S1), a Type II methanotroph (Methylosinus trichosporium OB3b) and the type X methanotroph (Methylococcus capsulatus (Bath)). The authors demonstrated that all the methanotrophs assimilated ammonia by the GS/GOGAT pathway. Type II methanotrophs were shown to

utilize the GS/GOGAT pathway exclusively, whether grown on  $N_2$ , nitrate or ammonia. Type I methanotrophs and the Type X methanotroph also possessed ADH and/or GDH. The pathway for assimilation depended on the growth substrate. Cells grown on nitrate (and in the case of Methylococcus capsulatus (Bath),  $N_2$ ) assimilated ammonia by the GS/GOGAT pathway, whereas cell grown on ammonia utilized ADH or GDH pathways. GS has been isolated from Methylococcus capsulatus (Bath) (Murrell & Dalton, 1983c) and was shown to be regulated by adenylation/deadenylation and by feedback inhibition. The gene encoding glutamine synthetase in Methylococcus capsulatus (Bath) has been sequenced (Cardy & Murrell, 1990). Comparison with GS genes from both bacteria and eukaryotes revealed that the M. capsulatus gene was conserved in five regions. These five regions had been shown previously to be conserved among all GS genes sequenced (Jannsen et al, 1988).

In addition to fixed nitrogen sources such as nitrate and ammonia, some methanotrophs possess the ability to fix dinitrogen. All Type II methanotrophs and the Type X Methylococcus capsulatus (Bath) can fix nitrogen, whereas the Type I methanotrophs so far described cannot (Murrell & Dalton, 1983a).  $N_2$ -fixation is the ability to convert molecular nitrogen ( $N_2$ ) to ammonia, a reaction catalysed by the nitrogenase enzyme complex. Possession of  $N_2$  fixing ability is investigated by  $^{15}N$  incorporation, or by following the reduction of acetylene.  $N_2$ -fixation is a widespread phenomena and is present in Azotobacter, Klebsiella, Bacillus, Clostridium and cyanobacteria, as well as countless other bacteria. It is essentially an anaerobic process and only occurs in bacterial cultures which are in a reduced  $O_2$  atmosphere. Ammonia and other fixed nitrogen sources act as

inhibitors of nitrogenase, indicating that  $N_2$ -fixation only occurs in conditions of fixed nitrogen starvation.  $N_2$ -fixation in methanotrophs was first observed by Davis et al (1964), although the acetylene reduction assay to detect  $N_2$ -fixation was not successful (Whittenbury et al, 1970a). This was explained when ethylene, the product of acetylene reduction, was observed to be a substrate for methane monooxygenase (Debont & Mulder, 1974; Dalton & Whittenbury, 1975) and that acetylene itself was toxic to methanotrophs (Dalton & Whittenbury, 1976). This inhibition was overcome by the addition of methanol to the reaction (Dalton & Whittenbury, 1975; 1976).

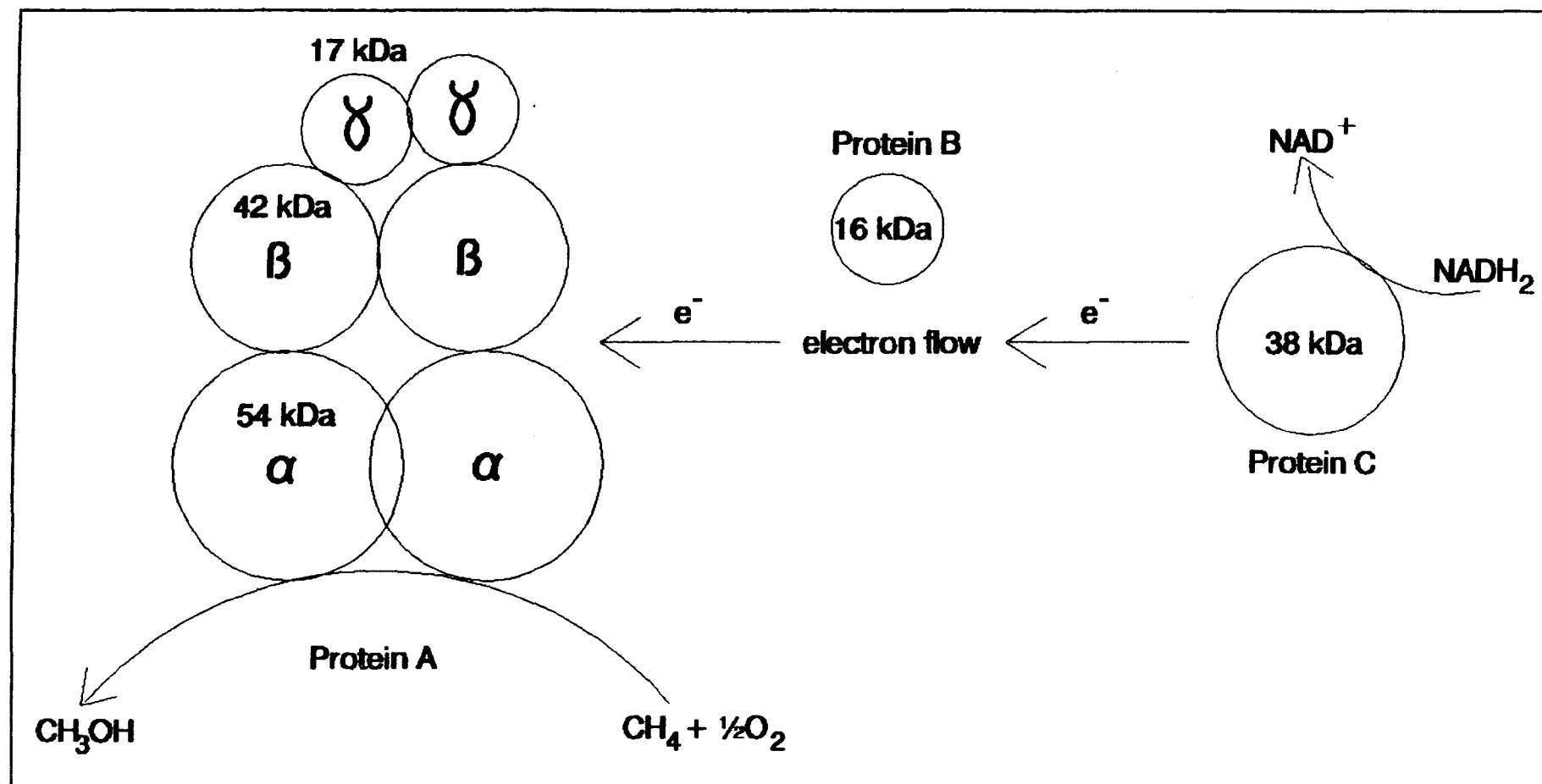
The genetic control of  $N_2$ -fixation has been studied in some detail and is reviewed by Dixon (1988). The genes concerned with  $N_2$ -fixation in Klebsiella are found clustered in an operon (the nif gene cluster), whereas the nif genes in other  $N_2$ -fixation bacteria studied appear to be spread about the chromosome. The nif genes from several methanotrophs have been detected and cloned (Methylosinus by Toukarian & Lidstrom, 1984a; 1984b; Methylococcus capsulatus (Bath) by Oakley & Murrell, 1991). A range of methanotrophs were screened with nifH, the gene which encodes the iron protein of nitrogenase (Oakley & Murrell, 1988). The results from this work showed that division of methanotrophs on the basis of their  $N_2$ -fixing ability was complicated at the genetic level. The Type I methanotrophs Methylomonas methanica and Methylobacter capsulatus showed homology with nifH, although they lack the ability to fix nitrogen. Other Type I methanotrophs tested showed no homology. As expected from their ability for  $N_2$ -fixation, all Type II methanotrophs and the Type X methanotroph Methylococcus capsulatus (Bath) exhibited good homology with the nifH gene probe in

conditions of high stringency. This indicates that possibly some or all Type I methanotrophs once possessed N<sub>2</sub>-fixing ability, but have since lost that ability.

## 1.6 Molecular biology of methanotrophs

### 1.6.1 Methane monooxygenase

Methane monooxygenase (MMO) is the enzyme which catalyses the oxidation of methane to methanol (fig 1.2). It is found in two forms, a membrane bound particulate form (pMMO) and a soluble cytoplasmic form (sMMO). All methanotrophs so far investigated possess a particulate methane monooxygenase (Dalton et al, 1990). In addition, some Type II methanotrophs possess a soluble MMO. Both forms of the enzyme are also present in the Type X methanotroph Methylococcus capsulatus (Bath). Whilst there is little information available regarding the particulate methane monooxygenase, much is known about the soluble form. The enzyme has been studied in detail in three bacteria: Methylococcus capsulatus (Bath) (Colby & Dalton, 1978), Methylosinus trichosporium OB3b (Fox et al, 1989) and Methylobacterium CRL-26 (Patel, 1984). The structure of the soluble methane monooxygenase is similar in all three systems (fig. 1.10) and has recently been reviewed (Dalton et al, 1990). Protein A is the hydroxylase component and has a molecular weight of approximately 220 kDa and consists of three subunits of 54 kDa, 42 kDa and 17 kDa arranged in an ( $\alpha, \beta, \gamma$ )<sub>2</sub> arrangement. Protein B is a regulatory protein (but appears to be lacking in Methylobacterium CRL-26) and has a molecular weight of around 16 kDa. Protein C is an iron-sulphur flavoprotein with a molecular weight of 42 kDa. It catalyses the transfer of reducing



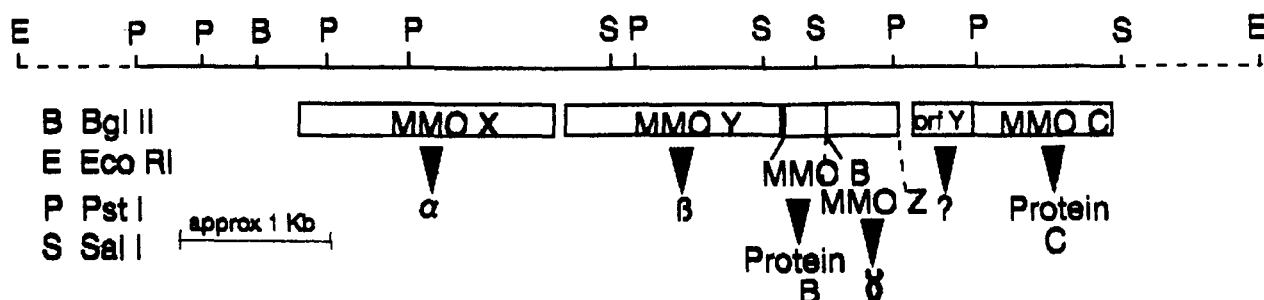
**Fig. 1.10:** Electron transfer in soluble methane monooxygenase. Subunit  $\alpha$  of protein A is the probable site for methane binding, but the actual arrangement of the subunits is unknown.



equivalents from NADH to Protein A.

The genes encoding the soluble methane monooxygenase enzyme complex have been isolated and cloned in Methylococcus capsulatus (Bath) (Stainthorpe et al, 1989; 1990a) and Methylosinus trichosporium OB3b (Cardy et al, 1991a; 1991b). In both organisms, the genes encoding the three subunits of protein A and proteins B and C are clustered as illustrated in fig 1.11a and 1.11b. Sequence analysis of both regions of Methylococcus capsulatus (Bath) and Methylosinus trichosporium OB3b show a high degree of similarity at the amino acid level (fig. 1.12).

Total DNA from a number of methanotrophs has been probed with part of the MMO gene cluster from Methylococcus capsulatus (Bath) (Stainthorpe et al, 1990b). Southern blot analysis shows that methanotrophs known to contain the soluble methane monooxygenase enzyme also show strong homology with the Methylococcus capsulatus (Bath) gene probe. Methanotrophs known to possess only the particulate form of methane monooxygenase showed no homology with the Methylococcus capsulatus (Bath) sMMO gene probe. This indicates that the sMMO gene probe from Methylococcus capsulatus (Bath) could be used to selectively detect soluble MMO in environmental isolates. Stainthorpe et al (1990b) demonstrated this by colony hybridization with culture collection strains and newly isolated, uncharacterized strains. A proportion of the newly isolated strains showed homology with the sMMO gene probe from Methylococcus capsulatus (Bath). These colonies were subsequently shown to possess sMMO, demonstrating that the sMMO gene probe can be used to detect sMMO in uncharacterized isolates.



**Fig. 1.11a: Soluble methane monooxygenase gene cluster in *Methylococcus capsulatus* (Bath)**

		Methylococcus		Methylosinus	
		bp	Da	bp	Da
mmoX	( $\alpha$ )	1581	60,636	1578	59,999
mmoY	( $\beta$ )	1164	44,726	1185	45,051
mmoZ	( $\gamma$ )	513	19,844	509	19,300
mmoB	(protein B)	426	16,020	417	14,885
orfY	(?)	312	11,943	309	11,985
mmoC	(protein C)	1047	38,581	1023	37,995

**Fig. 1.11b: Comparison between the protein products of the sMMO gene cluster from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b**

*Methylosinus trichosporium* OB3b  
*Methylococcus capsulatus* (Bath)

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30
M A I S L A T K A A T D A L K V N R A P V G V E P Q E V H K
| | : | | | | | | | | : | | : | | | |
M A L S T A T K A A T D A L A A N R A P T S V N A Q E V H R

60
W L Q S F N R D F K E N R T K Y P T K Y H M A N E T K E Q F
| | | | | : | | : | | | | | : | | | | |
W L Q S F N W D F K N N R T K Y A T K Y K M A N E T K E Q F

90
K V I A K E Y A R M E A A K D E R Q F G T L L D G L T R L G
| : | | | | | | | | : | | | | |
K L I A K E Y A R M E A V K D E R Q F G S L Q V A L T R L N

120
A G N K V H P R W G E T M K V I S N F L E V G E Y N A I A A
| | : | | | : | | | | : | | | | |
A G V R V H P K W N E T M K V V S N F L E V G E Y N A I A A

150
S A M L W D S A T A A E Q K N G Y L A Q V L D E I R H T H Q
| : | | | | : | | | | |
T G M L W D S A Q A A E Q K N G Y L A Q V L D E I R H T H Q

180
C A F I N H Y Y S K H Y H D P A G H N D A R R T R A I G P L
| | : | | : | | : | | : | | | | |
C A Y V N Y Y F A K N G Q D P A G H N D A R R T R T I G P L

210
W K G M K R V F A D G F I S R D A V E C S V N L Q L V G D T
| | | | | | | | : | | | | |
W K G M K R V F S D G F I S G D A V E C S L N L Q L V G E A

240
C F T N P L I V A V T E W A I G N G D E I T P T V F L S V E
| | | | | | | | : | | | | |
C F T N P L I V A V T E W A A A N G D E I T P T V F L S I E

270
T D E L R H M A N G Y Q T V V S I A N D P A S A K F L N T D
| | | | | | | | : | | | | |
T D E L R H M A N G Y Q T V V S I A N D P A S A K Y L N T D

300
L N N A F W T Q Q K Y F T P V L G Y L F E Y G S K F K V E P
| | | | | | | | : | | | | |
L N N A F W T Q Q K Y F T P V L G Y L F E M G S K F K V E P

330
W V K T W N R W V S E D W G G I W I G R L G K Y G V E S - R
| | | | | | | | : | | | | |
W V K T W N R W V Y E D W G G I W I G R L G K Y G V E S P R

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360
V L R D A K R D A Y W A H H D L A L A A Y A M W P L A F A R
| : | | | : | | | | | | | | : | | : | |
S L K D A K Q D A Y W A H H D L Y L L A Y A L W P T G F F R

390
L A L P D E E D Q A W F E A N Y P G W A D H Y G K I F N E W
| | | | : | : | : | | | | | | : | : |
L A L P D Q E E M E W F E A N Y P G W Y D H Y G K I Y E E W

420
K K L G Y E D P K S G F I P Y Q W L L A N G H D V Y I D R V
| : | | | : | | | | | | | | : | : |
R A R G C E D P S S G F I P L M W F I E N N H P I Y I D R V

450
S Q V P F I P S L A K G T G S L R V H E F N G K K H S L T D
| | | | : | : | : | | | | : | | : |
S Q V P F C P S L A K G A S T L R V H E Y N G Q M H T F S D

480
D W G E R Q W L I E P E R Y E C H N V F E Q Y E G R E L S E
| | | | : | | | : | | | : | | | : |
Q W G E R M W L A E P E R Y E C Q N I F E Q Y E G R E L S E

510
V I A E G H G V R S D G K T L I A Q P H T R G D N L W T L E
| | | | : | | | : | | | : | | | : |
V I A E L H G L R S D G K T L I A Q P H V R G D K L W T L D

527
D I K R A G C V F P D P L A K F -
| | | | : | | | : | | | : |
D I K R L N C V F K N P V K A F N

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Fig. 1.12a: Homology between the deduced amino acid sequence of the  $\alpha$ -subunit of protein A of sMMO from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath). Sequence data obtained from Cardy et al (1990a) and Stainthorpe et al (1990a).

Homology between OB3b and MCB = 94%

*Methylosinus trichosporium* OB3b  
*Methylococcus capsulatus* (Bath)

```

30
M S Q P Q S S Q V T K R G L T D P E R A A I I A A A V P D H
| | | | | : : : | | | | | : | : | : | : | : | : | :
M S M L G E R - - - R R G L T D P E M A A V I L K A L P E A

60
A L D T Q R K Y H Y F I Q P R W K P L S E Y E Q L S C Y A Q
| | | | | : : : | | | | | : | : | | | : | : | | | |
P L D G N N K M G Y F V T P R W K R L T E Y E A L T V Y A Q

90
P N P D W I A G G L D W G D W T Q K F H G G R P S W G N E S
| | | | | : : : | | | | | : | : | | | : | : | | | |
P N A D W I A G G L D W G D W T Q K F H G G R P S W G N E T

120
T E L R T T D W Y R H R D P A R R W H H P Y V K D K S E E A
| | | | | : | : | | | : | : | | | : | : | | | : | : |
T E L R T V D W F K H R D P L R R W H A P Y V K D K A E E W

150
R Y T Q R F L A A Y S S E G S I R T I D P Y W R D E I L N K
| | | | | : | : | | | : | : | | | : | : | | | : | : |
R Y T D R F L Q G Y S A D G Q I R A M N P T W R T S S C N R

180
Y F G A L L Y S E Y G L F N A H S S V G R D C L S D T I R Q
| : | : | : | : | : | : | : | : | : | : | : | : | : |
Y W G A F L F N E Y G L F N A H S Q G A R E A L S D V T R V

210
T A V F A A L D K V D N A Q M I Q M E R L F I A K L V P G F
| : | : | : | : | : | : | : | : | : | : | : | : | : |
S L A F W G F D K I D I A Q M I Q L E R G F L A K I V P G F

240
D A S T D V P K K I W T T D P I Y S G A R A T V Q E I W Q G
| : | : | : | : | : | : | : | : | : | : | : | : | : |
D E S T A V P K A E W T N G E V Y K S A R L A V E G L W Q E

270
V Q D W N E I L W A G H A V M I A T F G Q F A R R E F F Q R
| | | | | : : | | | | | | | | | : | | | | | | | |
V F D W N E S A F S V H A V Y D A L F G Q F V R R E F F Q R

300
L A T V Y G D T L T P F F T A Q S Q T Y F Q T T R G A I D D
| | : | : | : | : | : | : | : | : | : | : | : | : |
L A P R F G D N L T P F F I N Q A Q T Y F Q I A K Q G V Q D

330
L F V Y C L A N D S E F G A H N R T F L N A W T E H Y L A S
| : | : | : | : | : | : | : | : | : | : | : | : | : |
L Y Y N C L G D D P E F S D Y N R T V M R N W T G K W L E P

360
S V A A L K D R V G L Y A K V E K S R A D R S E E E L R G A
| : | | | : | : | : | : | : | : | : | : | : | : | :
T I A A L R D F M G L F A K L P A G T T D K E E I T A S L Y

390
A A S S A I G R S I T P D K I G F R V D V D Q K V D V L A G
| : | : | : | : | : | : | : | : | : | : | : | : | : |
R V V D D G S R T T P A G S T S R R T A I R - - - - S L K

Y - K N
: : :
F W Q D

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*Methylosinus trichosporium* OB3b  
*Methylococcus capsulatus* (Bath)

```

30
M A K R E P I H D N S I R T E W E A K I A K L T S V D Q A T
| | | | | : | : | : | : | : | : | : | : | : | : | :
M A K L G - I H S N D T R D A W V N K I A H V N T L E K A A

60
K F I Q D F R L A Y T S P F R K S Y D I D V D Y Q Y I E R K
| : | : | : | : | : | : | : | : | : | : | : | : | : |
E M L K Q F R M D H T T P F R N S Y E L D N Y L W I W E A K

90
I E E K L S V L K T E K L P V A D L I T K A T T G E D R A A
| | | | | : | : | : | : | : | : | : | : | : | : | :
L E E K V A V L K A R A F N E V D F R H K T A F G E D A K S

120
V E A T W I A K I K A A K S K Y E A D G I H I E F R Q L Y K
| : | : | : | : | : | : | : | : | : | : | : | : | : |
V L D G T V A K M N A A K D K W E A E K I H I G F R Q A Y K

150
P P V L P V N V F L R T D A A L G T V L M E I R N T D Y Y G
| | : | : | : | : | : | : | : | : | : | : | : | : |
P P I M P V N Y F L D G E R Q L G T R L M E L R N L N Y Y D

T P L E G L R K E P G V K V L H L Q A -
| | | | | : | : | : | : | : | : | : | : | : | : | :
T P L E E L R K Q R G V R V V H L Q S P H

```

Fig. 1.12c: Homology between the deduced amino acid sequence of the  $\beta$ -subunit of protein A of sMMO from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath). Sequence data obtained from Cardy et al (1990a) and Stainthorpe et al (1989).

Homology between OB3b and MCB = 85%

Fig. 1.12b: Homology between the deduced amino acid sequence of the  $\beta$ -subunit of protein A of sMMO from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath). Sequence data obtained from Cardy et al (1990a) and Stainthorpe et al (1989).

Homology between OB3b and MCB = 83.5%

*Methylosinus trichosporium* OB3b  
*Methylococcus capsulatus* (Bath)

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                                     30
M S S A H N A Y N A G I M Q K T G K A F A D E F F A E E N Q
| | : | | : | | : | | : | | : | | : | | : | | : | | : |
H S V N S N A Y D A G F M G L K G K D F A D Q P F A D E N Q

                                     60
V V H E S N A V V L V L M K S D E I D A I I E D I V L K G -
| | | | : | | | | : | | | | : | | | | : | | | | : | | |
V V H E S D T V V L V L K K S D E I N T F I E E I L L T D Y

                                     90
G K A K N P S I V V E D K A G F W W I K A D G A I E I D A A
| : | | : | | : | | : | | : | | : | | : | | : | | : |
K K N V N P T V N V E D R A G Y W W I K A N G K I E V D C D

                                     120
F A G E I L G K P F S V Y D L L I N V S S T V G R A Y T L G
| : | | : | | : | | : | | : | | : | | : | | : | | : |
F I S E L L G R Q F N V Y D F L V D V S S T I G R A Y T L G

                                     150
T K F T I T S E L M G L D R A L T D I - -
| : | | : | | : | | : | | : | | : | | : | | : | | : |
N K F T I T S E L M G L D R K L E D Y H A

```

Fig. 1.12d: Homology between the deduced amino acid sequence of protein B of sMMO from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath). Sequence data obtained from Cardy et al (1990a) and Stainthorpe et al (1989).

Homology between OB3b and MCB = 89.4%

Fig. 1.12e: Homology between the deduced amino acid sequence of protein C of sMMO from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath). Sequence data obtained from Cardy et al (1990a) and Stainthorpe et al (1990a).

Homology between OB3b and MCB = 85%

*Methylosinus trichosporium* OB3b  
*Methylococcus capsulatus* (Bath)

```

                                     30
M - - - Y Q I V I E T E D G E T C R R M R P S E D W I S R A
| : | : | : | : | : | : | : | : | : | : | : | : | : |
H Q R V H T I T A V T E D G E S L R F E C R S D E D V I T A

                                     60
E A E R N - - L L A S C R - A G C A T C K A D C T D G D Y E
| : | : | : | : | : | : | : | : | : | : | : | : | : |
A L R Q N I F L M S S C R E G G C A T C K A L C S E G D Y D

                                     90
L I D V K V Q A V P P D E E E D G K V L L C R T F P R S D I
| : | : | : | : | : | : | : | : | : | : | : | : | : |
L K G C S V Q A L P P E E E E E G L V L L C R T Y P K T D I

                                     120
H L L V P Y T Y D R I S F E A I Q T N W L A E I L A C D R V
| : | : | : | : | : | : | : | : | : | : | : | : | : |
E I E L P Y T H C R I S F G E V - G S F F A E V V G L N W V

                                     150
S S N V V R L V L Q R S R P M A A R I S L N F V P G Q F V D
| : | : | : | : | : | : | : | : | : | : | : | : | : |
S S N T V Q F L L Q K R P D E C G N R G V K F E P G Q F M D

                                     180
I E I P G T H T R R S Y S M A S V A E - D G Q L E F I I R L
| : | : | : | : | : | : | : | : | : | : | : | : | : |
L T I P G T D V S R S Y S P A N L P N P E G R L E F L I R V

                                     210
L P D G A F S K F L Q T E A K V G M R V D L R G P A G S F F
| : | : | : | : | : | : | : | : | : | : | : | : | : |
L P E G R F S D Y L R N D A R V G Q V L S V K G P I G V F G

                                     240
L H D H G G R S R V F V A G G T G L S P V L S M I R Q L G K
| : | : | : | : | : | : | : | : | : | : | : | : | : |
L K E R G M A P R V F V A G G T G L A P V V S M V R Q M Q E

                                     270
A S D P S P A T L L F G V T N R E E L F Y V D E L K T L A Q
| : | : | : | : | : | : | : | : | : | : | : | : | : |
W T A P N E T R I Y F G V N H E P E L F Y I D E L K S I E R

                                     300
S M P T L G V R I A V V N D D G G N G V D K G T V I D L L R
| : | : | : | : | : | : | : | : | : | : | : | : | : |
S M R N L T V K A C V W H P S G D W E G E Q G S P I D A L R

                                     330
A E L E I - - - - D L L L G H A R R R R R R R E T A R S C R
| : | : | : | : | : | : | : | : | : | : | : | : | : |
E D L E S S D A N P D I Y L C G P P G M - - - I D A A C -

                                     360
E D H R D R C P A W R S D F L E K F L A S G - -
| : | : | : | : | : | : | : | : | : | : | : | : | : |
E L V R S R G I P G E Q V F F E K F L P S G A A

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### 1.6.2 Methanol dehydrogenase

Methanol dehydrogenase is the enzyme responsible for the oxidation of methanol to formaldehyde. In Gram negative methanotrophs, this enzyme is a dye-linked (pyrrolo-quinoline quinone, or PQQ) protein first described in Pseudomonas M27 by Anthony & Zatman (1964a, b). The enzyme appears to be present in all Gram negative methanotrophs. The following description is taken from Anthony (1986; 1990). Methanol dehydrogenase (MDH) is situated in the periplasm and comprise two subunits; one of 60 kDa which carries a non-covalently bound PQQ prosthetic group and a subunit of 8.5 kDa. The subunits are arranged in a  $(\alpha, \beta)_2$  arrangement. The pH optimum of the enzyme in vitro is generally pH 9.0 or higher and the enzyme is often stable at pH 4.0. The protein accounts for between five and fifteen per cent of soluble protein when induced by methylotrophic growth. The enzyme has a broad substrate range for alcohols and oxidizes most primary alcohols. Enzyme activity requires an electron donor which is generally the acidic cytochrome  $c_1$ . This is oxidised by a Class I c-type cytochrome  $c_1$ . This is the substrate for the membrane bound terminal oxidase. During the oxidation of methanol to formaldehyde energy is produced by proton translocation.

Genetic studies show that the methanol dehydrogenase system is coded for by a number of genes (Lidstrom & Stirling, 1990; Lidstrom 1990; Lidstrom, 1991). Methylobacterium extorquens AM1 contains a minimum of 20 genes involved in methanol dehydrogenase activity (Nunn & Lidstrom, 1986a, 1986b; Lidstrom, 1991) whilst in Methylobacterium organophilum XX, at least fourteen genes have been identified (Machlin et al, 1988). The genes are found in several clusters and the organis-

ation of the genes in M. extorquens AML and M. organophilum XX are similar (fig 1.13). Functions have been ascribed to several of the genes. Genes have been identified which code for the methanol dehydrogenase large subunit structural gene, moxF, the cytochrome  $c_1$ , moxG and the small subunit structural gene, moxI. The moxF gene from several methylotrophs has been sequenced (Paracoccus denitrificans by Harms et al, 1987; Methylobacterium organophilum XX by Machlin & Hanson, 1988; Methylobacterium extorquens AML by Anderson et al, 1990) and partial sequence of Methylomonas species A4 by Waechter-Brulla & Lidstrom, 1990 is available. The sequence data show that the moxF gene is highly conserved between species (fig 1.14).

In Gram positive methylotrophs, methanol oxidation is not catalysed by the periplasmic PQQ-linked methanol dehydrogenase found in Gram negative bacteria. There are a variety of enzymes found in the Gram positive methylotrophs. In Bacillus, for example, a PQQ-independent NAD-dependent methanol dehydrogenase was found (Arfman et al, 1989). This has been studied in detail (Arfman et al, 1989; 1991). The enzyme was situated in the cytoplasm and appears to donate electrons to the electron transport chain at or above the level of cytochrome  $b$ . The enzyme has a relatively low affinity for methanol but the bacteria compensate by producing high levels of enzyme (up to 22% of soluble cell protein. Methanol oxidation in Ammycolatopsis methanolica (previously known as Nocardia sp. 239) has recently been shown to proceed via a dye-linked dehydrogenase (van Ophem et al, 1991). This enzyme has been found to use the tetrazolium dyes 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) and p-iodonitro-tetrazolium as electron acceptors in vitro. Enzyme assays in cell-free

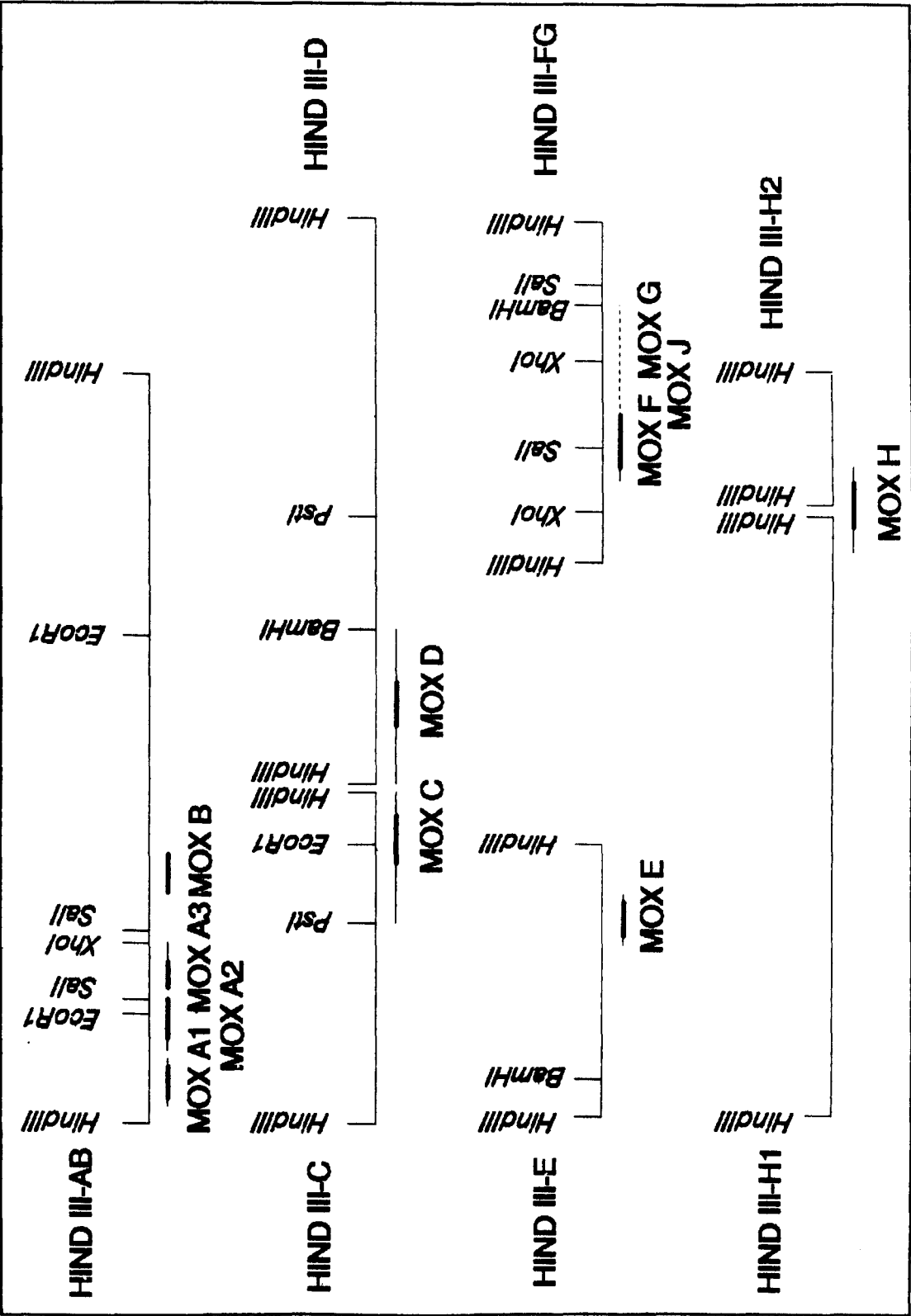


Fig. 1.13 : Methanol oxidation genes - complementation classes of *Methylobacterium extorquens* AM1 (after Nunn & Lidstrom, 1986)



*Methylobacterium extorquens* AM1  
*Methylobacterium organophilum* XX  
*Paracoccus denitrificans*

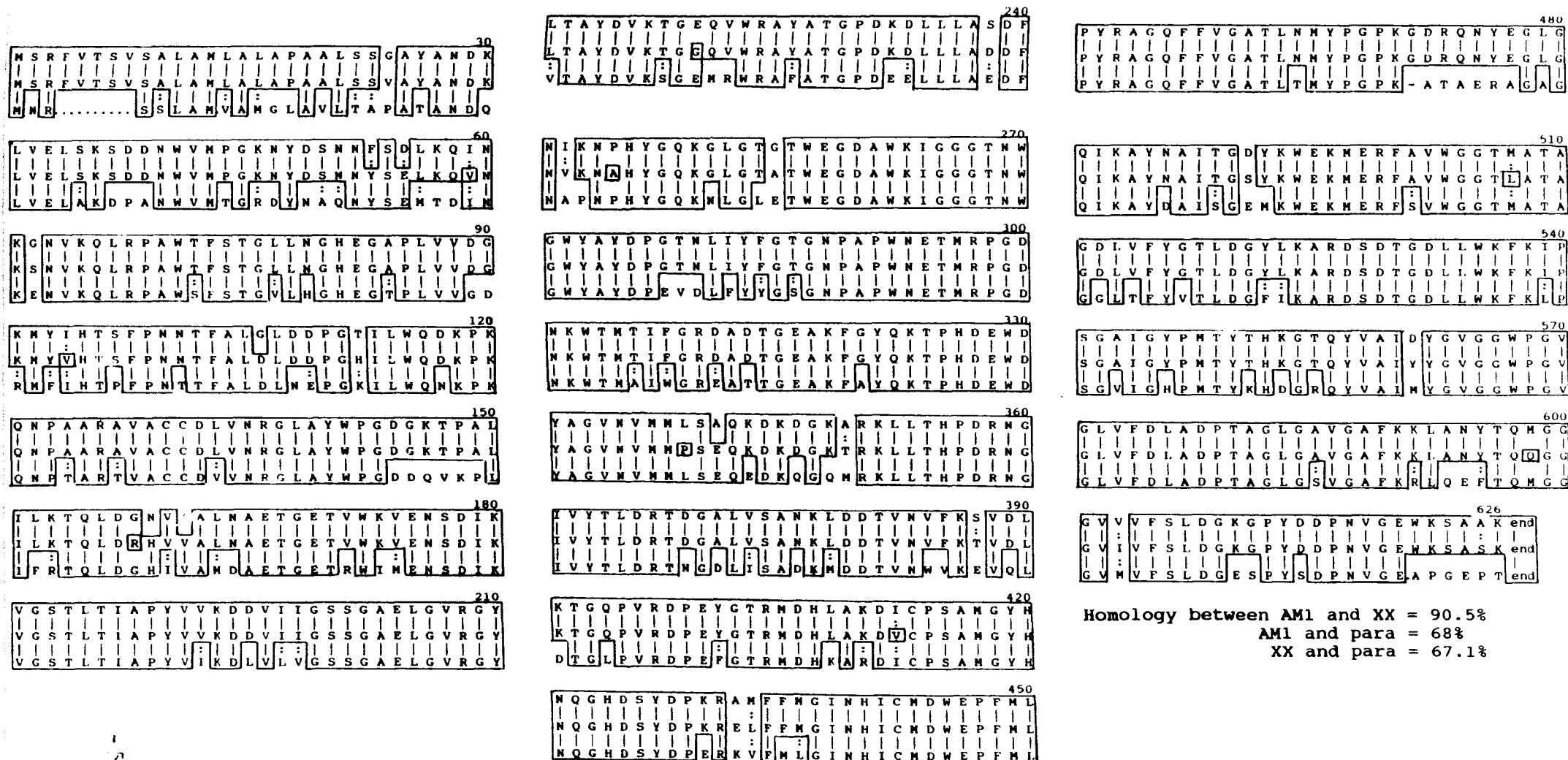


Fig. 1.14: Homology between the deduced amino acid sequence of methanol dehydrogenase structural gene *moxP* from three methylotrophs - *Methylobacterium extorquens* AM1, *Methylobacterium organophilum* XX and *Paracoccus denitrificans*. Sequence data was obtained from Anderson et al (1990), Machlin & Hanson (1988) and Harms et al (1987).

extracts indicated that the enzyme was cytoplasmic. Methanol-utilizing yeast oxidize methanol by a different system to those described above. This involves an alcohol oxidase and is described in section 1.1.2.

### 1.7 Detection of bacteria in the natural environment

Quantitative information on bacterial numbers and rates of growth, activity, and cycling of nutrients is essential for an understanding of the complex interactions between bacteria in natural environments. Detection methods can be as simple as counting bacterial cells to the more specific detection of unique nucleotide sequences. The following methods are based, unless stated otherwise, on detection of bacteria in aquatic samples, but the methods could be easily adapted to soil or sediment bacteria.

#### 1.7.1 Direct counts of bacteria

Attempts to obtain direct counts of bacteria can be divided into two approaches. The total number of bacteria in a sample can be counted microscopically, with no regard to the proportion of viable cells present (that is cells which contribute to the bacterial activity) or bacteria can be cultivated to calculate viable cells, or cells filling a specific nutritional niche. Both approaches are unsatisfactory for a number of reasons and these are discussed below.

Direct counting of bacteria after staining with a fluorescent dye is a common and reliable method of obtaining total numbers of bacteria in a sample. The principle for all of the many published methods is similar. Samples must be fixed immediately after collection with formaldehyde or glutaraldehyde. They can then be stored after fixation for

several weeks. The bacteria in a small sample (1-10 ml) are stained with a fluorescent dye, and filtered onto a black membrane filter. The filter is then viewed under a fluorescent microscope. The filters may be cellulose based or polycarbonate (Jones, 1979) and both are equally suitable. The membranes are stained black with Dylon no. 8 Ebony black (Jones, 1979), Irglan Black (Hobbie et al, 1977), Lanasyn brilliant black (Fry & Davies, 1985) or Sudan Black B (Zimmerman et al, 1978). Commercially supplied black membranes are available, but staining with the above dyes proves to be more satisfactory (Jones, 1979). The fixed sample is stained by one of a number of stains. Acridine orange (Hobbie et al, 1977) and 4'6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980; Coleman, 1980) are the most commonly used stains. The stained samples are filtered under vacuum onto the black filters and examined under a fluorescent microscope. Many fluorescent microscopes are suitable for visualising the cells (Fry, 1988). The bacteria are then counted and the number of cells in one millilitre is calculated. Recently, Schallenberg et al (1989) described an adaptation of DAPI staining to enumerate bacteria in sediment samples.

Before the introduction of methods using fluorescent stains, bacteria were stained with erythrosin. After filtration onto membrane filters, the bacteria were viewed under phase contrast or bright field microscopy (Fry, 1988). It is hard to distinguish between bacteria and detritus of a similar size by using this method but this can be overcome if the bacteria are viewed on agar by phase contrast microscopy. Most debris has a similar contrast to the agar and will not be observed whilst the bacteria are clearly seen. The samples must be concentrated by centrifugation (Fry & Zia, 1982). Centrifugation may mean

that certain bacteria such as small bacteria, or those with low density may not pellet and small bacteria are also hard to visualise under phase contrast microscopy. Direct counting under phase contrast can be a useful method when direct counts of bacteria are needed and there is no access to a fluorescent microscope.

Flow cytometry has recently been used for detection of aquatic bacterial populations (Robertson & Button, 1989; Sieracki et al, 1985 Tyndall et al, 1985). Bacteria are stained by fluorescent dyes (e.g. DAPI) or by fluor labelled antibodies and are detected by flow cytometry. This allows information on population cell size and DNA content (Robertson & Button, 1989). Naturally fluorescent bacteria can also be counted using flow cytometry, and their proportion in a population calculated.

Counting bacteria microscopically as described above does not discriminate between viable cells and non-viable cells (Roszak & Colwell, 1987). Cells in a dormant but potentially viable state may not take up stains, whereas non-viable cells may be stained but do not contribute to the bacterial activity of the ecosystem. This could be overcome by direct plating techniques. Although plate counts for total bacterial numbers are considered inadequate, bacterial groups can be detected by direct plating onto selective media. There are many reviews on selective media for bacteria, for example Schneider and Rheinheimer (1988). Samples of water (or soil suspensions) are either directly spread onto agar plates or filtered through a membrane filter under vacuum and the filter placed onto agar plates. The sample may need to be diluted if the selected bacteria are in high numbers. Selective media can be used to detect, for example, chitin- and

cellulose-decomposers, nitrogen fixing bacteria, denitrifying bacteria, sulphate-reducing bacteria, luminescent bacteria, photosynthetic bacteria and a range of bacteria with specific nutrient requirements. The drawback of selecting bacteria on solid agar plates is that many bacteria do not grow on solid media. This problem has been recognised for many years (Jones & Mollison, 1947) and whilst selective plating allows isolation of representative organisms, it has recently been estimated that only 1% or less of naturally occurring bacteria can be cultured by conventional laboratory techniques (Fuhrman et al, 1988; Somerville et al, 1989), so these methods do not allow for an estimation of bacterial numbers.

Postgate et al (1961) described a method for determining the number of viable bacteria in a population without plating the sample onto agar plates by 'slide culture'. Bacterial cells are mixed with agar and placed within a brass ring, which rests on a microscope slide. A coverslip is placed over the agar, and the slide is incubated for two to four hours. After this time, viable cells are reported to have undergone at least one division. There are obvious drawbacks to this method. Some cells have a division time of considerably longer than this time. Also bacteria which are sensitive to agar will appear as non-viable by this method. It does, however, provide a convenient technique to monitor bacterial populations of cultures known to survive on agar, for example when examining the effect of antimicrobial compounds on bacterial cultures.

The most probable number (MPN) method is a technique for enumerating viable organisms by successive dilution of the sample and statistical analysis (e.g. see Melchiorri-Santolini, 1972). A dilution

series is made of the sample (usually ten replicates for each dilution). These are incubated with any nutritional additions necessary and scored after incubation as positive or negative for growth. The pattern of positive and negative scores is analysed statistically to estimate the most probable number of bacteria in the original sample. This method avoids the use of solid media but is laborious and less accurate than other methods.

#### 1.7.2 Measurement of bacterial biomass in aquatic samples

Whereas the above section could be described as direct detection of bacteria, the following methods are indirect measurements of bacterial biomass. The measurement of the biomass of a bacterial community indicates the energy stored in that population. Biochemical assays are the most effective way of calculating the biomass, as this excludes mineral mass and detritus which can affect direct calculation of biomass.

Adenosine triphosphate (ATP) has been used extensively as a biomass indicator. ATP measurements reflect the biomass of living organisms, as it is rapidly lost after death of the organisms. Although it is so popular, it is an indicator of total biomass, not a specific bacterial measurement. Filtration with a nylon mesh will remove large zooplankton, but algal cells are more difficult to separate from bacteria. It is useful, however, in environments with few or no algae. Samples must be examined soon after collection to ensure that the ATP measured represents the environmental state. The samples are filtered to separate the bacteria from ATP in the water. The ATP is then extracted from the cells. There are many extraction

methods used and they fall into five groups: boiling buffer solutions, inorganic acids, organic acids, organic solvents and inorganic bases. One of the most popular methods uses boiling Tris (tris-hydroxymethyl-aminomethane hydrochloride) buffer. The extracted ATP is measured, usually by calculating the light emitted from addition of luciferin and luciferase to the ATP. Luciferase catalyses the conversion of luciferin and ATP to adenosine monophosphate, an inorganic pyrophosphate, a product and light. The ATP content in aquatic samples can be converted to cell carbon measurements (Hamilton & Holm-Hansen, 1965). ATP content varies with the nutritional and physiological state of the cell. An adaptation of this method is to measure the total adenylate pool, that is adenosine tri-, di- and monophosphate. This is described by Karl & Holm-Hansen (1978). This measurement is not dependent on the nutritional or physiological state of the cell, and allows an estimation of both the biomass and the total cell numbers of a population.

Other biomass indicators that can be measured to give an estimate of bacterial population include phospholipids. A description of phospholipid biomass measurements is made by Fry (1988). Chlorophyll a and bacteriochlorophylls are used as biomass indicators for photosynthetic organisms. Chlorophyll a is found in eukaryotic and prokaryotic cells, and is a general photosynthetic organism biomass indicator. Bacteriochlorophylls provide a photosynthetic prokaryotic biomass indicator. Chlorophylls are extracted from samples into solvents. Boiling or cold methanol and cold 90% acetone are commonly used for the extraction stage (Fry, 1988). The amount of chlorophyll present is measured by spectrophotometry and calculated statistically (Stal et al, 1984).

Cell wall components are also used as bacterial biomass indicators. As there are differences between the cell walls of Gram positive and Gram negative bacteria, there are few components that can be usefully employed to estimate bacterial biomass. Lipopolysaccharide measurements allow a calculation of the biomass of Gram negative bacteria as lipopolysaccharide is not present in significant amounts in Gram positive organisms. Clean, unpolluted waters contain high numbers of Gram negative bacteria (80-95%, Watson et al, 1977), but polluted waters and sediments contain more Gram positive organisms, and this method is not suitable in these environments. The most sensitive method is a turbidometric assay described by Watson et al (1977). Muramic acid is present in all bacterial cell walls, but is present in higher amounts in Gram positive bacteria than in Gram negative bacteria and is in very high levels in cyanobacteria. In order to use muramic acid as a biomass indicator, an estimate of numbers of Gram positive and Gram negative bacteria is needed. There are several methods for measuring muramic acid in sediments, for example; enzymic conversion to lactate (Moriarty, 1980); chemical analysis (King & White, (1977) and acid hydrolysis (Fazio et al, 1979), but the methods are complex and are not sensitive enough to measure the low levels of muramic acid in water samples (Fry, 1988).

### 1.7.3 Measurement of bacterial activity

Bacterial activity measurements reflect the numbers of metabolically active bacteria in a sample. The synthesis of various cell components is generally measured by following the incorporation of a radiolabelled substrate. From the rates calculated, an estimate of the



total bacterial activity of a sample is obtained. The activity of a sample does not indicate bacterial numbers, but when used in conjunction with the direct counting techniques, the physiological state of a population can be determined.

The most commonly used substrate is [methyl- $^3\text{H}$ ] thymidine ( $^3\text{TdR}$ ). Thymidine is incorporated into DNA and measurement of  $^3\text{TdR}$  incorporation reflects DNA synthesis and hence the bacterial activity. Thymidine uptake is generally restricted to prokaryotes (Grivell & Jackson, 1968) and as such, measurements of thymidine incorporation can be directly related to prokaryote activity. The method used to determine the incorporation of  $^3\text{TdR}$  into bacterial DNA was first developed by Fuhrman & Azam (1980; 1982; Fuhrman et al, 1980). The popularity of the method reflects its simplicity. The following procedure is based on Fuhrman & Azam (1980) as modified by Joint & Pomroy (1987). Water samples are incubated with  $^3\text{TdR}$ . At the end of the incubation period (from thirty minutes to 2 hours) the DNA is precipitated by addition of ice cold trichloroacetic acid. The sample is filtered and the incorporation of tritiated thymidine is calculated from the radioactivity as measured by a liquid scintillation counter. Thymidine incorporation is only useful as a measurement of bacterial production if several assumptions are made (Carman et al, 1988). Incorporation of the label must be linear with time and due only to bacterial uptake; radioactivity of the DNA must result from direct incorporation of the thymidine and not from recycling of degradation products of thymidine catabolism; thymidine should not be incorporated into other cellular macromolecules. Fuhrman & Azam (1982) demonstrated by autoradiography that organisms labelled with tritiated thymidine

were bacteria. Experiments also showed that 65-80% of the trichloroacetic acid insoluble material collected on the filter is DNA (Fuhrman & Azam, 1982). Carman et al (1988) and Brittain & Karl (1990), among others, argue that thymidine is incorporated into macromolecules other than DNA and as such is an unreliable method for detecting bacterial activity. It has also been demonstrated that some bacteria cannot take up thymidine (Pollard & Moriaty, 1984; Jeffrey & Paul, 1990) including some chemolithotrophic bacteria (Johnstone & Jones, 1989). DNA synthesis could be underestimated if unlabelled thymine was incorporated into the cell (Jeffrey & Paul, 1988; Ellenbroek & Cappenburg, 1991). Even with the drawbacks associated with this method, it still remains the primary method for measurement of bacterial activity. The incorporation of tritiated  $^3\text{TdR}$  into DNA can be accurately estimated by examining DNA from the sample (Jeffrey et al, 1990), and not the trichloroacetic acid insoluble material, which may contain other macromolecules.

Other radiolabelled substrates are used to determine bacterial activity. Commonly used substrates include tritiated glucose (Li, 1984; Geider, 1989), tritiated acetate (Stanley & Staley, 1977), adenine (Karl, 1980, 1982) and  $^{14}\text{C}$ -leucine (Kirchman et al, 1985; 1986; Chin-Leo & Kirchman, 1988). LaRock et al (1988) compared the bacterial activity by pulse-labelling bacteria with a variety of labelled substrates, including adenine, uridine and iododeoxyuridine (a thymidine analogue) to label DNA or RNA, and  $^{32}\text{P}$  to label phospholipids in the cell membrane. Pulse labelling is claimed to provide different samples with equal amounts of radioactive label (LaRock et al, 1988) and that adenine to label nucleic acids and  $^{32}\text{P}$

to label phospholipids are the most successful pulse labels.

Metabolic inhibitors can be used to demonstrate bacterial activity. Nalidixic acid is one such compound. This causes an inhibition of cell division, but cell growth is unaffected. The cells therefore enlarge. Nalidixic acid is only effective on Gram negative bacteria, but by combining it with other antibiotics, cells which appear enlarged under microscopic analysis are deemed to be metabolically active (O'Carroll, 1988).

Bacterial activity within particular bacterial groups can be determined by the biological oxygen demand, or BOD. Water samples are placed into sealed bottles, the substrate of interest is added, and the oxygen concentration measured before and after incubation and compared to a control which contains no added substrate. This method has been described for methane and hydrogen-oxidizing bacteria and Thiobacilli by Sorokin (1972).

#### 1.7.4 Immunodetection of bacteria

Detection of microbes in the soil by antibodies raised to antigens unique to that microbe was first described by Schmidt & Bankole (1962; 1965) who fluorescently labelled antibody raised against Aspergillus flavus. This was closely followed by fluorescent-antibody detection of Bacillus in soil samples by Hill & Gray (1967) and the detection of Rhizobium by Schmidt et al (1968). The use of polyclonal or monoclonal antibodies raised to whole cells are surprisingly specific, showing little cross-reactivity between related species. Antibodies can be raised against whole cells or against specific products. Those used for detection of whole cells are generally

polyclonal and are raised in rabbits. Whole cells are harvested and resuspended in a buffered saline solution before injection in rabbits. Antibody production in the rabbit builds up and blood is taken after approximately two weeks. Red blood cells are separated from the serum by centrifugation. The serum containing the antibody is conjugated with a fluorescent dye and bacteria in the samples probed with the antibody.

The most common fluorescent stain used to label antibodies is fluorescein isothiocyanate (Reed & Dugan, 1978; Hoff, 1988; Volsch et al, 1990). Aquatic samples to be probed are filtered onto dark filters under vacuum and soil samples are prepared as smears. Labelled antibody is incubated with the sample and binds to the antigen on the bacteria. An alternative indirect staining technique is to bind unlabelled antibody to the sample and detect the antibody with labelled antiserum against the antibody (for example anti-rabbit antiserum) (Reed & Dugan, 1978). Stained bacteria are counted under a fluorescent microscope in the same way as fluorescent labelled bacteria. Immunofluorescence labelled bacteria can also be detected by flow cytometry. Nitrosomonas was detected by flow cytometry in this way and the numbers counted compared to most probable number (MPN) estimates of ammonia-oxidizing bacteria from activated sludge samples over a six month period (Volsch et al, 1990). The numbers counted by flow cytometry and the activity of all ammonia-oxidizers rose and fell in parallel over this period.

Antibodies can be indirectly labelled with radioactively labelled antiserum (Arredondo & Jerez, 1989) and with non-fluorescent dyes (Hawkes et al, 1982). Labelled bacteria are detected by a 'dot-immuno-

binding assay'. Radioactive labelling (Arredondo & Jerez, 1989) is achieved by utilizing  $^{125}\text{I}$ -protein A or an  $^{125}\text{I}$ -labelled antisera to the first antibody. Cells are applied directly to a membrane filter in a 'dot-blot' array, the antibody is bound to the cells and the radio-labelled antisera or protein A is attached to the antibody. This method was a sensitive and specific method for detection and enumeration of Thiobacillus ferrooxidans (Arredondo & Jerez, 1989). Dot-immunobinding assay methods can be adapted to use an indirect peroxidase labelled antibody (Hawkes et al, 1982). After the cells are applied to a membrane filter and bound to the specific antibody as above, the filter is incubated with a peroxidase conjugated second antibody against the specific bacterial antibody. The peroxidase is reacted with 4-chloro-1-naphthol and hydrogen peroxide. A positive antibody reaction will appear blue after development of the colour.

Immunodetection of bacteria is a sensitive and accurate technique for detecting numbers of particular groups of bacteria. When used in conjunction with methods for counting the total bacterial population in an environment it can indicate the proportion of bacteria that react with the antibody. One problem that is apparent with immunodetection is that antibodies can only be raised to previously isolated bacteria. In an environment where most bacteria are thought to be non-culturable by existing methods, this does not reveal any more about the bacteria in an environment than selective culture methods. The antibodies used must also be carefully screened. Any cross-reactivity with other bacteria will lead to an over-estimation of the bacterial group tested.

#### 1.7.5 Bacterial detection by nucleic acid hybridization

The application of nucleic acid hybridization techniques to the detection of bacteria in the environment is of increasing importance. Nucleic acid hybridization offers several advantages over more traditional techniques, such as immunoassays, biomass studies and traditional culture methods. These advantages include the detection of bacteria that are not readily culturable and cannot be biochemically defined, identification of bacteria that are taxonomically distinct but related by a metabolic process and the detection of genetic markers, such as antibiotic resistance genes. The latter point is an important aspect in the calculation of gene transfer rates in studies on the release of genetically engineered organisms. There have been many recent reviews on the detection of bacteria in the environment by nucleic acid probes (Hazen & Jimenez, 1988; Lidstrom, 1990; Ogram & Saylor, 1988; Pickup, 1991; Walker & Dougan, 1989 among others). Gene probes for detection can be divided into two classes: those that are species-specific, and those that are function-specific. Species-specific probes are generally developed from genes encoding ribosomal RNA whereas the function-specific probes examine the community for the potential to perform a given function. These two classes of probes are discussed below in sections 1.7.7 and 1.7.8.

Nucleic acid probing involves hybridization of a probe to bacterial DNA. Two techniques of DNA hybridization are employed in environmental samples: colony hybridization and hybridization of total DNA extracts. Colony hybridization requires that bacteria are cultured before hybridization. This means that "hard-to-culture" bacteria are excluded from the hybridization. Total DNA isolation methods extract

DNA from bacteria in a sample without cultivation. Ideally, the extraction techniques would isolate DNA from every single bacterium in a sample, but in practise this is not feasible. The best techniques extract DNA from a representative proportion of the bacteria in the sample without enforcing any selection. Total bacterial DNA extraction methods are described in detail in section 1.7.6.

After DNA extraction, either directly from a sample or from colonies, the DNA is transferred to a hybridization membrane. Colonies are frequently cultured on membranes placed on top of the growth medium. This allows nutrients to pass through and when the colonies have grown sufficiently, they are lysed and the DNA is fixed onto the hybridization membrane. DNA can be applied onto nitrocellulose in a 'dot-blot' array (Kafatos et al, 1979). Dot-blot techniques have the advantage that large numbers of samples can be processed simultaneously and dilute DNA samples are concentrated by filtration through the membrane. Membrane-bound DNA is denatured and the filter is prehybridized with denatured eukaryote DNA (for example, salmon testis DNA) to block sites of non-specific DNA binding. The probe is generally labelled with a radiolabelled nucleotide and is hybridized with the denatured sample DNA. Analysis by autoradiography or liquid scintillation indicates the presence or absence of the DNA in the sample and can also with careful controls estimate the numbers of target bacteria. Non-radioactive labels usually involve detection of the hybridized DNA by enzyme-linked or fluorescence-labelled antibodies. Enzyme-linked antibodies can be then detected by a colorimetric assay.

### 1.7.6 Extraction of nucleic acid from environmental samples

Bacterial DNA extracted from environmental samples must be relatively pure for DNA hybridization techniques. Impurities in environmental samples are generally due to contamination from organic compounds in the environment such as humic acids and phenolics. DNA may be extracted directly from the bacterial population of an environment or from cultured colonies. Colony extraction is the easier of the two methods. The colonies can be transferred from the original cultivation medium to filters. They can then be lysed and the DNA fixed. The DNA is ready for hybridization immediately.

Direct extraction of DNA from environmental samples must be essentially non-selective. Lysis techniques used should not favour certain bacterial species over others. As mentioned above, harvesting bacteria, especially from aquatic samples, should not bias certain groups of organisms. Direct extraction of DNA has been performed on both soil and sediment samples and on a range of aquatic samples. Soil and sediment samples frequently have high concentrations of organic impurities and the DNA needs to be carefully purified. Aquatic samples contain a lower concentration of bacteria and large samples may need to be processed before enough DNA is extracted. A method for amplification of small quantities of DNA is described below in section 1.7.9.

To isolate DNA from soil bacteria, two approaches have been employed. Firstly, the bacterial cells are removed from soil particles prior to DNA extraction. Holben et al (1988) developed a method in which soil was removed from the bacteria by homogenization with polyvinylpolypyrrolidone (PVPP) and low speed centrifugation. The



bacteria were then lysed with lysozyme and the DNA was partially purified using ammonium acetate and ethanol. Finally, the DNA was purified by hydroxyapatite column chromatography, as described by Ogram et al (1988). The second approach involves isolation of DNA from bacteria which are not separated from the soil before DNA extraction. Steffan et al (1988) compared the two approaches. DNA extraction from bacteria isolated from soil was performed as described above. Lysis of bacteria which were still bound to soil particles was carried out by adding a detergent and disrupting the cells with a bead beater. PVPP was added to remove humic material. After further extraction the DNA was purified by caesium chloride/ethidium bromide density gradient. Steffan et al (1988) concluded that the direct lysis method produces a greater quantity of DNA. However, the DNA obtained from both methods was of sufficient purity to allow hybridization.

DNA is extracted from aquatic bacteria using environmental samples that have been concentrated. This is achieved by either filtration or by centrifugation. Centrifugation can impose a size selection on the bacteria harvested. Centrifugation methods are relatively faster to perform, however, and once collected the cells are lysed in a similar manner as for soil bacteria. Filtration can overcome the size selection if the pore size of the filter is chosen with care. To remove eukaryotic DNA and to prevent rapid filter blockage, Fuhrman et al (1988) suggested prefiltering the sample through a glass fibre filter and then harvesting the bacteria by a second filtration step through a 0.22  $\mu$ m pore size nucleopore filter. Although the final yield of DNA is low it was reported to show no selection for any bacterial group. Somerville et al (1989) extracted

aquatic bacterial DNA by filtration through a commercial filtration unit, Sterivex-GS. Within this filter unit, Somerville and coworkers performed all the DNA extraction procedures. Both of these methods resulted in pure DNA suitable for DNA manipulation techniques such as restriction enzyme analysis.

RNA extraction from environmental samples is important for two reasons. Firstly, extraction of messenger RNA (mRNA) allows an investigation into gene expression and the presence of specific proteins whereas detection of homologous DNA sequences shows the potential for that gene to be expressed in an environment. mRNA hybridization indicates the level of expression of that gene in the environment. Secondly, ribosomal RNA extraction is important in the development of species-specific probes and this is described in section 1.7.7. Tsai et al (1991) describe a method for obtaining mRNA from seeded soils. The mRNA obtained allowed identification of two gene transcripts by DNA:RNA hybridization.

#### 1.7.7 Nucleic acid hybridization-ribosomal RNA probes

Ribosomal RNA sequences have highly conserved regions between taxonomically related bacteria, and are less well conserved as the evolutionary distance becomes greater. Ribosomal RNA sequences offer a useful tool for evolutionary and ecological study for several reasons (Olsen et al, 1986). They are found in all living organisms and contain regions conserved throughout all kingdoms and more diverse regions which show species specificity (Giovannoni et al, 1988). The rRNAs are ancient molecules and are extremely conserved in their structure. This makes them readily identifiable. Ribosomal RNAs are a

significant component of cell mass and are readily recovered from many organisms. There appears to be no transfer of rRNA between species, so the evolutionary relationships between organisms can be calculated. In bacteria, there are three rRNA species, 5S (approximately 120 nucleotides), 16S (1600 nucleotides) and 23S (3000 nucleotides). The degree of similarity between different bacterial species can be calculated by a comparison of the 16S rRNA sequences. Statistical analysis allows evolutionary trees to be compiled (Olsen et al, 1986; Woese, 1987) and this has been performed for many bacterial species and groups (archaebacteria by Amils et al, 1989; Fibrobacter by Amman et al, 1990a; Legionella by Fry et al, 1991; Frankia by Hahn et al, 1990; mycobacteria by Teske et al, 1991; methylotrophs by Tsien et al, 1990, and many others).

Ribosomal RNA can be selectively sequenced using a method described by Weller & Ward (1989). 16S rRNA sequences are cloned and sequenced from cDNA synthesised from the 16S rRNA molecules. The specificity is obtained by use of an oligonucleotide from a conserved region of the 16S rRNA molecule. Similar methods have been used by Lane et al (1985) and Ward et al (1990). An alternative method used involves polymerase chain reaction amplification and sequencing of the amplification product (Weisburg et al, 1991) (see section 1.7.9 for full description of polymerase chain reaction).

Ribosomal RNA sequence analysis has allowed characterization of the bacterial species in many environments containing bacteria which have not been characterized by more traditional means. These include the marine environment (Britschgi & Giovannoni, 1991; Giovannoni et al, 1990; Pichard & Paul, 1991), hot springs in Yellowstone National

Park (Stahl et al, 1985; Ward et al, 1990; Weller & Ward, 1989), bacterial endosymbionts (Amman et al, 1991) and ruminal bacteria (Stahl et al, 1988). Ribosomal RNA sequence detection has the ability for specific, accurate and simple identification of bacteria in natural environments. Enumeration of bacteria by 16S rRNA has recently been attempted (Amman et al, 1990b). By labelling the rRNA probe with a fluorescent marker, bacteria can be detected by flow cytometry. Although this work was performed on laboratory cultures, it demonstrated how an individual cell could be detected microscopically when labelled in this manner. Using flow cytometry, levels of target cells could be detected at concentrations of 3% of the total number of cells. Labelling rRNA in this way, population drifts could easily be monitored.

Recent work has indicated that DNA-dependent RNA polymerase sequence may also be used to determine phylogenetic relationships between bacterial groups (Zillig et al, 1989). Initial results indicate similarities between the taxonomic data obtained from RNA polymerase sequence and those obtained from 16S rRNA sequences.

#### 1.7.8 Nucleic acid hybridization-gene probes

Much of the environmental probing work published to date using DNA probes other than rRNA sequences has concentrated on detection of plasmid-borne genes in genetically engineered microorganisms, to follow the fate of DNA after release into the environment. Due to the stringent controls on the release of these organisms, this work has primarily been carried out in microcosms (Amy & Hiatt, 1989; Deflaun & Paul, 1989; Sayler et al, 1985; Steffan & Atlas, 1988) and in hay

(Hendrick et al, 1991). Results from these studies show that the probe used for detection must be specific enough to avoid non-specific binding. When a broad-host range plasmid was used as a probe, high levels of non-specific binding were observed (Sayler et al, 1985). Detection of plasmids with narrow host ranges and therefore unlikely to transfer between bacteria, have been used to follow the fate of organisms in unnatural environments, for example E.coli containing a plasmid in lake water (Amy & Hiatt, 1989). The fate of bacteria inoculated into hay as preservatives has also been followed (Hendrick et al, 1991). In this study, the presence of a naturally occurring plasmid isolated from Bacillus pumilus was used as a molecular marker to estimate populations of plasmid-containing strains in treated and untreated hay.

Comparison between methods of detecting bacteria by probing colony blots and environmental DNA samples with specific DNA sequences and most probable number (MPN) analysis was carried out by Steffan et al (1989). Colony blots and MPN showed that both culture methods gave similar levels of sensitivity. When uncultured environmental DNA samples were probed, however, it was found that the sensitivity was greatly improved.

Colony blot methods have proved important in detection of pathogenic bacteria. Recent reports detail detection of Listeria (Kim et al, 1991) and Yersina (Nesbakken et al, 1991) in food by specific probes. These methods offer rapid and accurate diagnosis of bacterial contamination.

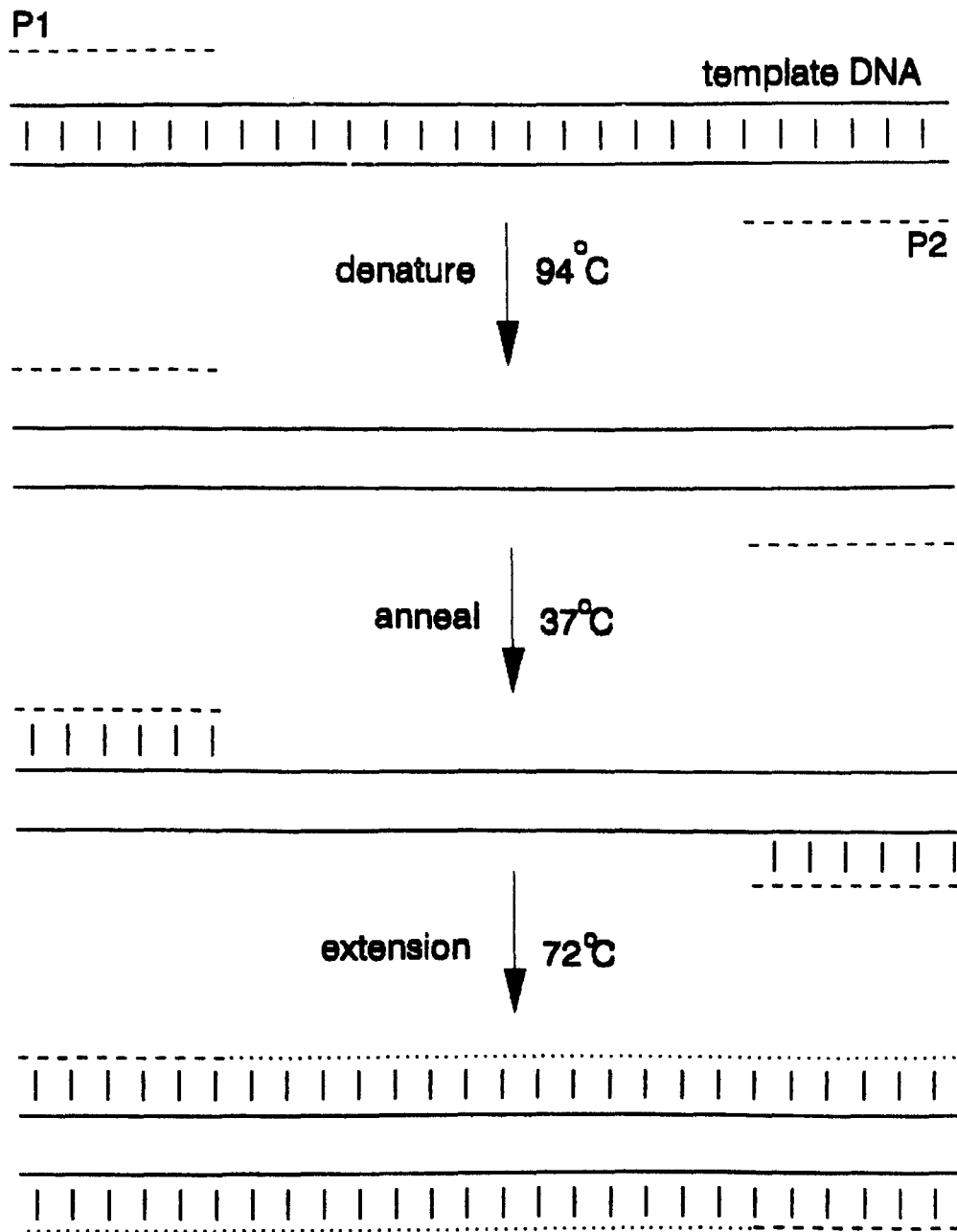
Many workers have used a functional gene to detect bacterial groups. Diagnostic gene probes have been described for many bacteria

and are the subject of a recently published book (Macario & de Macario, 1990). Selenska & Klingmuller (1991) detected nitrogen fixation genes in inoculated soil using a probe from Enterobacter agglomerans. The fate of E. agglomerans after inoculation into soil was followed by conventional plate techniques. DNA extracted from the soil was hybridized to the nitrogen fixation gene probe from Enterobacter. nif genes could be detected at a time when conventional plating techniques no longer gave rise to colonies. In this case, the Enterobacter is either becoming non-culturable, or the nitrogen fixation genes are transferred to indigenous non-culturable bacteria.

A species-specific and function-specific probe was developed by Wimpee et al (1991). These workers probed DNA from marine bacteria with luciferase gene probes. At low stringencies, the luciferase genes bound to DNA from a number of different bacterial species and could be described as function-specific. At higher stringencies, however, the gene probes were species-specific.

#### 1.7.9 The use of the polymerase chain reaction in environmental probing

The polymerase chain reaction (PCR) is a method of amplifying specific DNA sequences. It was first developed for use in medical diagnosis, but recently the methodology has been adapted to amplify small amounts of environmental DNA samples to give a DNA concentration detectable by hybridization techniques. PCR was first described by Saiki et al (1985) and is illustrated in fig 1.15. Oligonucleotide primers of approximately 20 bases were added to template DNA together with nucleotides and DNA polymerase from E. coli. Amplification was



**Fig. 1.15** Diagram of the polymerase chain reaction. Primers P1 & P2 are homologous with a region of the template DNA. After the template DNA is denatured, the primers bind to the separate strands. The DNA is extended from the primers by *taq* polymerase and the amount of template DNA doubles. The cycle is repeated many times. Temperatures given are only for illustration.

achieved by a cycle of denaturation of the template at a high temperature followed by annealing of the primers at a lower temperature and extension with DNA polymerase. This cycle is repeated many times (usually between 20 and 40). Each cycle theoretically doubles the amount of target sequence. Initial amplification procedures used DNA polymerase I which is unstable at the high denaturation temperatures. Polymerase was added after each denaturation step, a laborious process. The method was improved by the discovery of the thermostable DNA polymerase from Thermus aquaticus (Taq polymerase) (Saiki et al, 1988). By using Taq polymerase in the reaction, only one addition of polymerase at the start of the cycle is needed. Theoretically, PCR can amplify DNA from one target sequence in  $10^5$  to  $10^6$  cells, although in practice this may be slightly lowered.

Amplification by PCR of DNA sequences found specifically in target bacteria allows detection of very small amounts of target DNA. Smaller samples from the environment may be processed, thus offering an advantage in ease of handling. Even very small amounts of DNA (as low as 1 fg or less) can be amplified and detected by hybridization or visualisation by conventional methods such as agarose gel electrophoresis. The disadvantages of using PCR are experimental: the DNA needs to be very pure as contaminants will inhibit the Taq polymerase and, due to the sensitivity of the method, care must be taken to avoid contamination of the sample by target sequence from a source outside the environment from which it was taken. In spite of this, PCR has already joined the methods used in environmental detection, for example; 16S rRNA sequences contain regions that are conserved in a wide range of bacteria. These specific sequences have been used with



PCR to amplify 16S rRNA sequences for phylogenetic study (Weisburg et al, 1991). A second example is found in the amplification of luciferase genes by using conserved sequences within the gene as primers to amplify species-specific luciferase genes (Wimpee et al, 1991). Thirdly, a wide range of harmful bacteria in environmental samples have been detected using PCR. In this area of research, detection by PCR avoids cultivation of pathogens. Bacteria detected include Legionella by Bej et al, 1991; Listeria by Deneer & Boychuk (1991); Vibrio vulnificus by Hill et al, 1991; Mycoplasma by Palmer et al, 1990; and Staphylococcus by Wilson et al, 1991. PCR has also proved its use in detection of genetically engineered organisms (Steffan & Atlas, 1988), and has become an established method in bacterial detection.

### 1.8 Detection of methanotrophs in the natural environment

The detection of methanotrophs in natural environments by previous workers has been via three general approaches: bacterial isolation, measurement of methane oxidation and immunological detection. The isolation of methanotrophic bacteria has been discussed above, together with the problems associated with detection of bacteria that cannot be cultured by conventional methods. This section is concerned with methods for detecting methanotrophic bacteria by the remaining two methods.

#### 1.8.1 Mass balance calculations of methane oxidation rates

Reeburgh (1976) proposed a mathematical model to determine the rate of anaerobic methane oxidation in Cariaco Trench sediments based

on methane concentration gradients and estimates of diffusion rates. The profile for methane concentration against depth was determined for two stations in the Cariaco Trench in two ways. Firstly, the methane concentration was measured directly by gas chromatography. This was then compared to profiles calculated analytically. This approach only gave a general indication of methane oxidation because of the difficulties in measuring accurately diffusion coefficients.

#### 1.8.2 Measurement of methane concentration changes

Jannasch (1975) and Sansone & Martens (1978), among others, calculated the rate of methane oxidation in fresh water and marine water respectively by following the decreases in methane concentration in water samples incubated in glass bottles. This method is only effective if the rates of methane oxidation are rapid enough to detect changes within a few hours, thus avoiding unpredictable bottle effects from prolonged incubation (Rudd & Taylor, 1986). The bottle effect produces an artificially high number of bacteria due to rapid reproduction in the collection vessel. It is a particular problem when sampling seawater, because seawater lacks natural adsorptive surfaces and when placed into glass bottles, nutrients become concentrated by adsorption on the wall of the bottle. To overcome the problem of low oxygen/methane concentrations at the oxic/anoxic interface, where methane oxidation activity often occurs, Jannasch (1975) diluted samples from Lake Kivu with surface water to increase the concentration of dissolved oxygen and described the measurements for methane oxidation obtained from Lake Kivu water samples in terms of order of magnitude accuracy. Accuracy could also be affected if the water samples contained methanogens, bacteria producing methane.

### 1.8.3 Methane oxidation rates determined by radiolabelled methane

The rate of methane oxidation can be calculated by adding radiolabelled methane ( $^{14}\text{CH}_4$ ) to an environmental sample and following its conversion to carbon dioxide ( $^{14}\text{CO}_2$ ) and cell carbon over an incubation period of up to 24 hours. This principle has been applied to samples from several different environments: (Whalen et al, 1990, in soil; Lidstrom & Somers, 1984, in freshwater).

Marine environments have also been sampled, e.g by Griffiths et al, 1982, who investigated methane oxidation rates in the Southeastern Bering Sea, and more recently by Ward et al, 1987, who measured methane oxidation rates in the Cariaco Basin. This approach offers several advantages over previous methods. Because of its relative sensitivity, the incubation times can be shortened, minimising the bottle effect and it is also unnecessary to increase the oxygen and methane concentrations to abnormal levels which could affect the measurements obtained.

### 1.8.4 Methane oxidation rates determined by carbon isotope analysis

The methane produced by bacteria usually has a low  $^{13}\text{C}$  content (Rosenfield & Silverman, 1959). Similarly, methanotrophs preferentially oxidise  $^{12}\text{C}$ ; thus the methane residue is consequently enriched for  $^{13}\text{C}$ . Zyakun et al (1983) demonstrated that the rise in  $^{13}\text{C}$  is proportional to the methane oxidized in a system and that measurement of the change in  $^{13}\text{C}$  content could be used to calculate methane oxidation rates.

The methods for measuring methane oxidation rates detailed above can indicate whether or not methanotrophs are present in an

environment. They do not allow a means of enumerating methanotrophs, but give a measurement of methanotrophic activity. High methane oxidation rates do not necessarily indicate large numbers of methanotrophs: a sample of a few highly active methanotrophs would have oxidation rates of similar values as a sample containing many bacteria with low oxidation rates.

There is a further complication in using methane oxidation rates to estimate methanotroph activity. Ammonia-oxidizing bacteria Nitrosomonas europaea and Nitrosomonas oceanus have been shown to oxidize methane to carbon dioxide as a final product (Jones & Morita, 1983; Ward, 1987; Hyman et al, 1988 among others). This would affect measurement of methane oxidation rates. It has been demonstrated that methane is oxidized by Nitrosomonas oceanus to cell carbon and CO<sub>2</sub> (Ward, 1990) and that methane is a specific inhibitor of ammonia oxidation. Ward (1987) proposed that the role of methanotrophs in the marine environment could be at least partially fulfilled by ammonia-oxidizing bacteria.

#### 1.8.5 Immunological detection of methanotrophs

Reed & Dugan, 1978, first described a procedure for detecting methanotrophs in Cleveland Harbour, USA. Antibodies were raised against two methanotrophs from a culture collection. The antibodies showed no cross reactivity to other culture collection methanotrophs tested. By fluorescent labelling of the antibodies, environmental samples were probed with the antibodies. This method allowed an accurate record of the number of bacteria that cross-reacted with the antibodies. Further work by Gal'chenko et al, 1988, who examined

fourteen species of methanotrophs isolated from the Black Sea and also compared the numbers obtained with methane oxidation rates determined by following the uptake of  $^{14}\text{CH}_4$ . No correlation was found between the numbers of methanotrophs detected and methane oxidation rates. This could be due to cross-reactivity of the antibodies with species of non-methanotrophic bacteria, or could reflect the inaccuracy of methane oxidation rate measurements.

Detection of methanotrophs by antibodies allows a method for enumerating bacteria, but has the drawback that the detection is specific for the species against which the antibody was raised. In practise, detection of methanotrophs by antibody probing can only tell us about the organisms that have previously been cultured.

### 1.9 Marine bacteria

The marine environment comprises three interconnecting oceans, namely the Atlantic, the Pacific and the Indian, and many smaller seas. Together they make up 97% of water on the planet. The seas increase in depth from a shallow continental shelf to deep basins of 4,000 to 6,000 metres. There are also deeper trenches, which can be as deep as 11,000 metres in the Pacific Ocean. Tides, waves and storms cause much water movement and upwelling of cold nutrient rich water is induced by wind. Seawater contains many elements which can support the growth of microorganisms, but the main components are sodium and chloride. The major components of seawater are listed in table 1.4. Average values for sodium chloride concentration in open oceans lies between 32 and 38 ppt, although these values fall around coastal regions with fresh water from the land. Sea water is an extremely

<b>COMPONENT</b>	<b>CONCENTRATION (mg/kg of seawater at 35 ppt salinity)</b>
Calcium	412
Magnesium	1294
Nitrogen	15
Potassium	399
Silicon	2.9
Sodium	10760
Strontium	7.9
Bicarbonate	145
Boron	4.6
Bromide	67
Chloride	19350
Fluoride	1.3
Sulphate	2712

**Table 1.4:** Major components of oceanic water (from Austin, 1988).

heterogeneous environment. Some areas, such as the Sargasso and Weddell seas are nutrient rich and support large numbers of bacteria, whereas other areas are nutrient limited (Austin, 1988).

Seas have a large surface area and as such, solar energy plays an important part in determining the marine environment. Where light can penetrate the water, photosynthesis by photosynthetic microorganisms occurs. This leads to the production of oxygen in the water and this area is known as the photic zone or oxic zone. Below this area lies the anoxic or aphotic zone. The depth to which light penetrates is determined by the turbidity of the water. In clean, unpolluted areas with low bacterial growth, light can penetrate to 200 m or more. The temperature of the water remains generally uniform when compared with terrestrial and fresh water temperatures. Water in temperate and tropical regions ranges between 25°C to 32°C whilst at the Poles it is an average of 3.5°C. The variation at the Poles is greatest here, with water ranging from -1.7°C to 8°C. The solar energy also influences temperature of the oceans, and warmer water rises, forming a region where water temperature suddenly falls, the thermocline. The sediments of the oceans are important biologically. They are usually anoxic and contain large quantities of organic material derived from sinking of minute organic particles (marine snow).

Within this complex environment, a diverse range of bacteria are found. Most are similar to terrestrial or fresh water species of bacteria but are adapted to life in the marine environment. The majority of marine bacteria are Gram negative rods, with a higher proportion of Gram positive organism occurring in the sediments. Many marine bacteria produce pigments on solid media (primarily yellow,

orange or red). They generally have an absolute requirement for sodium chloride and strains may prefer to utilize amino acids rather than sugars.

Direct microscopic analysis of marine bacteria indicate that they are very small when compared with their terrestrial and fresh water counterparts and a sizable proportion of the bacteria appear to be non-culturable, at least by conventional techniques (Kogure et al, 1979). Several workers have explained the small size of marine bacteria as a starvation response (for example Novitsky & Morita, 1976; 1977; 1978) and when nutrients are added to the cells, an increase in size is apparent (Amy & Morita, 1983; Amy et al, 1983). Austin (1988) discusses the implications of these results. If the small bacterial cells are in a state of dormancy for several years (Amy & Morita, 1983; Amy et al, 1983) until higher levels of nutrients are available, can they be described as viable?

Microbiology of the deep sea, that is water below 1,000 m, has specific problems when compared with microbiology in general. Not only is it difficult to obtain samples from these depths, the bacteria which live in deep sea areas are very often sensitive to the lower pressures at the surface of the water. Bacterial numbers in the deep sea have been estimated by epifluorescent microscopy to be at a concentration of  $1.44 \times 10^8$  cells per litre (Deming, 1985). Taxonomically, deep sea bacteria resemble those found in surface waters, except in deep-sea hydrothermal vents. In these environments, there are many archaeobacteria, thermophiles, sulphur-oxidizers, chemolithotrophs and a variety of more unusual bacteria. These are



described in Austin (1988), Jannasch (1989) and Jannasch & Taylor (1984).

#### 1.10 Aims of the project

This study was undertaken in an attempt to begin to answer some fundamental questions about methane cycling in the marine environment. Methane is of increasing importance as global warming increases. As a 'greenhouse gas', methane concentrations affect global warming. To gain an insight into methane cycling might help to understand and predict global warming. The marine environment offers an ideal platform for the study of methane cycling. Firstly, sea water covers most of the planet. Secondly, methane dissolves readily in water. There has been some controversy as to whether the oceans are a source or a sink for methane. Very little is known about methane cycling in the marine environment. Methane oxidation by bacteria is an important part in methane cycling in any environment. In the marine environment, little is known about this process or the bacteria responsible for it. This study concentrates on examining the occurrence of the bacteria responsible for methane oxidation in one area of the marine environment. The rate of methane oxidation was compared with total bacterial activity and the concentration of methane. Representative organisms were isolated from another marine habitat to compare the marine bacteria with their fresh water counterparts. Finally, a method was developed to detect methanotrophs. This method relied on molecular techniques and was not limited by cultivation of methanotrophs or by measuring the rate of activity of the bacteria. This surmounts the problems associated with these methods as described above.

## METHODS AND MATERIALS

## 2.1 Media

All chemicals used were from Sigma, Fisons or Aldrich unless otherwise stated, and were of Analar grade. Radio-isotopes were obtained from Amersham Ltd., Bucks., England. The specific activity of each isotope is stated.

### 2.1.1 Growth of marine methanotrophs

Initial cultivation and maintenance of strains was carried out in autoclaved seawater. A supplement containing (per litre) 10 mg  $\text{NH}_4\text{Cl}$ ; 5 mg  $\text{Na}_2\text{HPO}_4$ ; 2.5 mg Fe-EDTA; 1.0  $\mu\text{g}$  vitamin  $\text{B}_{12}$ ; 1.0  $\mu\text{g}$  biotin; 100  $\mu\text{g}$  thiamine (NEM-1) (Sieburth et al; 1987) was added at a 1/1000 concentration after autoclaving. Solid media were routinely prepared by adding 1.5% w/v Noble agar to seawater before autoclaving (15 min at 15 psi), unless otherwise stated.

After initial enrichment, isolates were routinely grown in the mineral salts medium (MS) of Whittenbury et al; (1970), with the addition of 1.5% w/v sodium chloride. MS was supplemented with nitrogen in the form of either ammonium (0.1% w/v  $\text{NH}_4\text{Cl}$ ) (AMS); nitrate (0.1% w/v  $\text{KNO}_3$ ) (NMS) or a dual nitrogen source (0.05% w/v  $\text{NH}_4\text{Cl}$  + 0.05% w/v  $\text{KNO}_3$ ) (ANMS). The media were sterilised by autoclaving. A vitamin solution (Gest et al; 1983) (0.05% v/v of a x 200 stock) and sodium phosphate buffer (pH 7.6) (1% v/v of a 200 mM stock) were added after cooling. A semi-solid medium was made by the addition of 0.4% w/v Noble agar to mineral salts medium before autoclaving.

Routine growth was carried out in liquid culture. A 250 ml flask containing 50 ml medium with the addition of supplements and phosphates was inoculated with 0.5 ml of a culture in early stationary growth, which had an absorbance at 540 nm of approximately 1.0. The flask was sealed with a rubber "suba-seal" stopper, and 50 ml air withdrawn using a needle and syringe. This was replaced with 100 ml methane (British Oxygen Company Ltd., London) introduced via a sterile needle and syringe. The flask was incubated at 30°C on an orbital shaker (200 rpm) for two to three days.

For growth of larger quantities of bacteria, cultures were grown by one of two methods. Batch cultures were grown in 2 l 'quick fit' flasks, which contained 1 l of medium, with all additions, and sealed with a rubber 'suba-seal' stopper. This was inoculated with 10 ml of a fresh culture. 300 ml air were removed from the flask and replaced with approximately 350 ml methane. The flask was incubated at 30°C on an orbital shaker (200 rpm) for two to three days. Fermenter cultures were grown in an LH 500 series fermenter run as a fed-batch system. 800 ml medium were inoculated with 50 ml from a fresh culture which was in exponential growth, at an absorbance at 540 nm of 0.8. Methane and air were added continuously at rates of 80 ml.min<sup>-1</sup> and 40 ml.min<sup>-1</sup> respectively. Samples were removed from the fermenter at regular intervals and replaced with an equal volume of fresh medium.

Solid plates were inoculated by either streaking the colonies with a sterile loop, or by spreading 100µl culture with a glass spreader. Semi-solid plates were formed by first inoculating cool

(37°C) media (after the addition of vitamins and phosphates) with 0.1% v/v culture at  $10^{-5}$  dilution. The plates were then poured and left to set.

Plates were placed into a "Gas-pak" anaerobic jar, and gassed with methane from an inflated football bladder to give an approximate atmosphere of 50% methane in air.

Routine incubations were carried out at 30°C. Flasks were incubated on an orbital shaker at 200 rpm.

#### 2.1.2 Growth of laboratory strains of methanotrophs

Methanotrophs from the laboratory culture collection were grown on plates and in liquid in mineral salts media with the addition of ammonium and/or nitrate as described previously [Whittenbury et al; (1970)].

#### 2.1.3 Growth of Escherichia Coli

Cultures of E. coli were routinely grown on LB medium (Maniatis et al; 1982) (sterilised by autoclaving at 15 min at 15 psi). Solid LB medium was achieved with 1.5% (w/v) Bacto agar, added before autoclaving.

Plates were inoculated by either streaking from a single colony with a sterile loop, or by spreading 100 µl culture with a glass spreader. Liquid cultures were inoculated by taking a loopful of a fresh colony from a plate. Plates and flasks were incubated at 37°C. Flasks were incubated on an orbital shaker at 200 rpm.

#### 2.1.4 Harvesting Cells by centrifugation

All bacterial cultures were harvested by centrifugation. Unless otherwise stated, centrifugation was at 10,000 rpm (17700 x g)(500 ml centrifuge pots) or 20,000 rpm (48400 x g)(50 ml centrifuge tubes) for 20 min in a Beckman J2-21M/E centrifuge. The temperature was kept at 4°C. Cell pellets were washed in either 10 mM Tris HCl, pH 8.0 or 20 mM phosphate buffer, pH 7.6.

### 2.2 Enrichment methods

#### 2.2.1 Seawater samples

Seawater was collected in clean plastic containers from a number of sites and from a number of depths. Initial enrichment studies were performed on seawater from areas in Plymouth Sound, England. Water was collected from inshore and offshore water, from deep offshore water and from the Tamar estuary. Enrichment studies were also performed on water from the Mediterranean and from the South Atlantic.

#### 2.2.2 Enrichment of methane-, methanol-, and methylamine-utilizing bacteria in shake flasks

250 ml "quickfit" flasks containing 50 ml of seawater (with NEM-1 supplement) were used to enrich for C<sub>1</sub>-utilizing bacteria. For methanol and methylamine enrichments, the carbon source was added at a concentration of 0.1% (v/v). Flasks were then sealed with "suba-seal" stoppers. For methane enrichments, methane was added as detailed in section 2.1. The flasks were incubated at 30°C on an orbital shaker at 200 rpm for several days to two weeks until turbidity was observed. Turbid cultures were subcultured by inoculation into 250 ml flasks

containing 50 ml autoclaved seawater (with NEM-1) supplement. Carbon sources were added as before.

For the routine investigation of methanotrophic bacteria in water samples when isolation of the bacteria was not needed, 1 l undiluted water sample was placed into a 2 l "quickfit" flask, which was then stoppered. Methane was added as described in section 2.1.1 and the flask incubated for five days.

### 2.2.3 Enrichment of methane-utilizing bacteria in bottles

500 ml serum bottles filled almost to the neck with seawater containing NEM-1 supplement were sealed with "suba-seal" stoppers. 50ml air was withdrawn and replaced with 100 ml methane. Enrichments were performed in duplicate, with 0.1% v/v formaldehyde added to one bottle from each pair. It had previously been reported that the addition of formaldehyde to bottle enrichments enhanced the growth of methanotrophic bacteria (Sieburth et al; 1987). Bottles were then placed at room temperature, and at 30°C, both shaking and static, and left for several days, until turbid cultures were obtained.

### 2.2.4 Enrichment of methane-, methanol-, and methylamine-utilizing bacteria on filters

100 ml-500 ml seawater was filtered under suction through 0.22  $\mu$ m nitrocellulose filters, and the filters placed onto 1.5% (w/v) Noble agar/seawater plates. For methanol and methylamine enrichments, the carbon source was added at a concentration of 0.1% (v/v) to the cooled molten agar before pouring the plates. Methane enrichment plates contained no added carbon sources, and were incubated in a methane

atmosphere as described in section 2.1.

Filters were also placed into 250 ml flasks containing autoclaved seawater (with NEM-1 supplement), and carbon sources added as in section 2.2.2. Both plates and flasks were incubated at 30°C, flasks were placed on an orbital shaker as before. Enrichments were left for a week until colonies developed on plates, or turbidity was observed in flasks. Any resulting colonies or turbid cultures were used to inoculate 250 ml flasks containing autoclaved seawater as described in section 2.1.

#### 2.2.5 Fermenter enrichment of methane-utilizing bacteria

An LH Fermentation (Stoke Poges, Bucks., U.K.) 100 Series fermenter with a working volume of 800 ml was inoculated with 600 ml seawater. Stirring was at 60 rpm, and air and methane were continuously provided at rates of 50 ml.min<sup>-1</sup> and 80 ml.min<sup>-1</sup> respectively. The temperature was maintained at 30°C. Incubation continued for up to one week until turbidity was observed. Samples from the fermenters were used to inoculate 250 ml flasks containing 50 ml autoclaved seawater (with NEM-1 supplement) and incubated in the presence of methane at 30°C as described in section 2.1.

#### 2.3 Obtaining single colonies of isolates

A dilution series of the enrichment culture was made and used to inoculate 100 ml autoclaved seawater/0.4% (w/v) Noble agar, from which plates were poured. After incubating in a methane atmosphere at 30°C for several days, colonies appeared. Single colonies were picked with the aid of a plate microscope and sterile Pasteur pipettes and



resuspended in 100 µl of autoclaved seawater. This was used to inoculate a further 100 ml of seawater/0.4% (w/v) Noble agar as above and the colonies isolated as before. After isolating with a Pasteur pipette, single colonies were tested for purity by inoculating 5ml autoclaved seawater in a 25 ml flask, sealed with a "suba-seal" stopper. 5 ml air were removed and replaced, via a needle, with 10 ml methane. The flasks were incubated in a shaking water bath at 30°C for several days, until turbidity was observed. The purity of the culture was determined by high power (x1000) light microscopy.

## 2.4 Characterization of isolates

### 2.4.1 Light microscopy

High power oil immersion (x1000) phase contrast light microscopy was performed routinely on a Unilux II microscope to examine cell purity.

### 2.4.2 Growth of isolates on solid media

The growth of isolates on a range of solidifying media was tested. Concentrations of the solidifying agents are listed in table 2.1. All solid media were prepared by adding the agents to seawater and autoclaving (15 min at 15 psi), except where indicated. The media were poured into sterile plastic petri dishes and allowed to set. 100 ul of a dilution series from a liquid culture of an isolate were then spread with an alcohol flamed glass spreader and plates were incubated at 30°C in a sealed container in a methane atmosphere, as described in section 2.1.

<b>SOLIDIFYING AGENT</b>
Oxoid agar No. 1
Bacto-agar (Difco)
Lab M agar
Noble agar (Difco)
Agarose (types I and II)(Sigma, St Louis, Mo, USA)
Pluronic polyol F127 (Gardener & Jones, 1964)
Silica Gel (Funk & Krulwich, 1964)
Ludox (DuPont)
Noble agar + activated charcoal

*Table 2.1: Solidifying agents used to test the growth of IR1 and DR1.*

#### 2.4.3 Microaerophilic growth of isolates

Microaerophilic growth was tested by inoculating 100 ml ANMS/0.4% (w/v) Noble agar (after cooling to 37°C), and adding 10 ml inoculated medium to sterile 15 ml glass test tubes. The tubes were sealed with "suba-seal" stoppers, flushed with nitrogen, oxygen or left unflushed. Methane and air were added as listed in table 2.2. The tubes were flushed by introducing nitrogen or oxygen into the tube via a needle. The gas was allowed to flow into the tube for 5 min, displaced gas escaping through a second needle open to the atmosphere. 5 ml of the gas were removed from the tube and methane and air to a total volume of 10 ml were added. All conditions were tested in duplicate. Tubes were incubated at 30°C for three days, after which time a clear band of growth could be seen in the agar.

#### 2.4.4 Initial characterization of physical properties of isolates

Cell size of the isolates was determined by use of a calibrated graticule used under high power (x1000) light microscopy.

Biochemical tests to determine the presence of a capsule (Hiss method); Gram stain; oxidase and catalase determination; and flagella staining (Gray method) were carried out as described by Gerhardt et al (1981).

#### 2.4.5 Testing the nitrogen source of isolates

The nitrogen source used by isolates was tested by inoculating 50 ml media containing different nitrogen sources in a 250 ml flask. The flasks were sealed with "suba-seal" stoppers, and methane added as described in section 2.1. The flasks were incubated for three days.

	<b>FLUSHED</b>	<b>METHANE</b>	<b>AIR</b>
1+2	no	yes	no
3+4	N2	yes	no
5+6	N2	yes	yes
7+8	O2	yes	no
9+10	O2	no	no
11+12	N2	no	no
13+14	no	no	no
15+16*	no	no	no
17+18*	N2	yes	yes
19+20*	N2	no	no

**Table 2.2: Conditions set up to test for micro-aerophilic growth of IR1 and DR1. Tubes marked with \* contained MS medium, which contained no fixed nitrogen source, and were set up to investigate nitrogen-fixing ability in IR1 and DR1.**

Flasks showing growth were used to inoculate a second flask containing the same nitrogen source, to ensure that no contaminating nitrogen source was carried over from the first inoculant. Nitrogen sources tested were as follows: 0.1% (w/v) ammonium chloride; 0.1% (w/v) potassium nitrate; 0.05% (w/v) ammonium chloride plus 0.05% (w/v) potassium nitrate; 0.05% (w/v) ammonium chloride; 0.05% (w/v) potassium nitrate; nitrogen free media.

#### 2.4.6 Growth of isolates on single amino acids as nitrogen sources

0.1% (w/v) of a single amino acid was added to 250 ml flasks containing 50 ml MS media. 50 ml cultures were pelleted by centrifugation, the pellets resuspended in MS media to wash away all nitrogen traces and recentrifuged. This was repeated and the pellet resuspended in 5 ml MS. Flasks were inoculated with 100  $\mu$ l resuspended culture, sealed with a "suba-seal" stopper and methane injected. Growth of the cultures was examined after five days growth. Amino acids tested were as follows: L-glutamic acid; L-proline; L-valine; L-glutamine; L-methionine; L-arginine; L-asparagine; L-cysteine; L-serine; L-glycine; L-phenylalanine.

#### 2.4.7 Growth of isolates on alternative carbon sources

100  $\mu$ l culture was used to inoculate 50 ml NMS in 250 ml "quick-fit" flasks with a variety of carbon sources other than methane. Flasks were incubated at 30°C for five days, and were inspected for turbidity. Addition of carbon compounds was as follows: methanol (0.1%; 0.2%; 0.5%; 1%; 2% v/v); methane sulphonate (15 mM); dimethyl sulphide (2 mM); dimethyl disulphide (2 mM); methane phosphonate

(5 mM); trimethylamine (15 mM); dimethylamine (15 mM); monomethylamine (15 mM); proteose peptone (0.02% w/v); nitromethane (0.1% v/v).

#### 2.4.8 Growth of isolates on low-iron plates

Semi-solid media with a low concentration of iron were made by removing the iron salts from ANMS media, and using ultra pure ELGA water for all solutions. After autoclaving, 100 ml cooled medium was inoculated, plates were poured, and incubated in a methane rich atmosphere for five days, until colonies had developed.

#### 2.4.9 Growth of isolates on alternative ion donors to NaCl

Sodium chloride was replaced in 50 ml ANMS medium with related salts. Flasks were inoculated with 0.5 ml fresh culture, sealed with "suba-seal" stoppers, and methane injected. The flasks were incubated at 30°C for five days, and examined for growth. Salts used were as follows: magnesium chloride; sodium fluoride; lithium chloride; calcium chloride; potassium bromide; potassium chloride and were added at a concentration of 1.5% (w/v).

#### 2.4.10 Growth requirement of isolates for copper

ANMS medium made without the addition of copper was used to test the requirement of copper for growth. An inoculant of the isolates was sedimented by centrifugation and resuspended in flasks containing 50ml copper-free medium before inoculating flasks. Growth was examined after two days incubation in a methane atmosphere at 30°C.

#### 2.4.11 Antibiotic resistance profiles

The antibiotic resistance profiles were tested by addition of the antibiotic at an appropriate concentration to ANMS medium. After incubation at 30°C, resistance was determined by growth after three days. Addition of antibiotics was as follows: tetracycline (25  $\mu\text{g.ml}^{-1}$ ); gentamycin (10  $\mu\text{g.ml}^{-1}$ ); trimethoprim (30  $\mu\text{g.ml}^{-1}$ ) penicillin G (1 unit); Streptomycin (10  $\mu\text{g.ml}^{-1}$ ).

#### 2.4.12 Growth optima

The growth of both isolates at different temperature, pH and sodium chloride concentrations was investigated. In all cases, growth was in ANMS medium. For the temperature and pH measurements, sodium chloride was added to ANMS at a concentration of 1.5% (w/v). For pH and sodium chloride measurements, the growth temperature was 30°C. For temperature and sodium chloride measurements, the pH was kept at 7.6. pH was varied by addition of phosphate buffer at different pH. All flasks for a particular growth parameter were inoculated at the same time with the same size inocula from a rapidly growing culture. Methane was added as previously described and the flasks were incubated on orbital shakers shaking at a speed of 200 rpm. After the lag phase, 1 ml samples were withdrawn from the flasks with a sterile needle and syringe, and the absorbance at 540 nm recorded on a Pye Unicam SP1800 spectrophotometer. Measurements were repeated two-hourly during exponential growth. All growth experiments were repeated in duplicate.

## 2.5 Enzyme assays

The carbon and nitrogen assimilation pathways and related enzymes were investigated in both isolates. All enzyme assays were performed at 30°C unless otherwise specified. All spectrophotometric assays were performed on a Pye Unicam SP1800 UV recording spectrophotometer. The cuvette carriage of this instrument was thermally controlled by attachment to a Churchill water circulator. All assays were performed at 30°C, and were equilibrated at 30°C for 2 min before initiation, unless otherwise stated. For assays that recorded the change in absorbance over time, the measured rate was linear for at least 3 min. Assays were performed in duplicate or triplicate.

### 2.5.1 Preparation of cells for enzyme assays

Fermenter grown cells at an absorbance at 540 nm of approximately 2.0, or batch grown cells at an approximate absorbance at 540 nm of 1.5 were used for these experiments. Cells were harvested by centrifugation, resuspended in with phosphate containing 1.5% sodium chloride, reharvested by centrifugation and resuspended in 2ml phosphate containing 1.5% sodium chloride. Cell-free extracts containing soluble, cytoplasmic proteins, were prepared by passing the cells twice through a French pressure cell (Aminco, Silver Spring, USA) of 138 mPa. The membrane fraction was removed from the cell-free extract by centrifugation at 18000 rpm; 30 min, 4°C in an MSE High Speed 18 centrifuge. The cell-free extract was used immediately, and was kept on ice.



Whole cell assays were performed on cells grown as above for cell-free extracts, harvested and resuspended in 2ml as above, but were not passed through the French pressure cell.

### 2.5.2 Protein determination of cell-free extracts

The amount of protein present in the cell-free extracts was determined using the 'Biorad' assay. 100  $\mu$ l cell-free extract plus water was added to 5 ml of a 1 in 5 dilution of 'Biorad' solution, and the colour allowed to develop at room temperature for 10 to 30 min, and the absorbance at 595 nm was recorded. This was compared with readings obtained from a calibration curve of known amounts of bovine serum albumin over a range of 1-10  $\mu$ g.ml<sup>-1</sup>

### 2.5.3 Nitrogen assimilation pathway enzymes

Cell-free extracts for nitrogen assimilation enzyme assays were prepared from cells grown on both ANMS and NMS media.

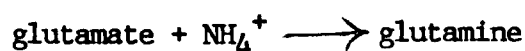
#### 2.5.3.1 Glutamine synthetase EC 6.3.1.2

Glutamine synthetase (GS) was assayed by three methods. Firstly, the reverse action of the enzyme was assayed as described by Bender et al (1977). This assay follows the formation of  $\gamma$ -glutamyl hydroxamate and ammonia from glutamine and hydroxylamine. Assays were performed in a final volume of 500  $\mu$ l which contained, in 135 mM imidazole-HCl pH 7.15; 18 mM hydroxylamine, 0.27 mM manganese chloride, 25 mM sodium arsenate pH 7.15, 40 mM ADP (sodium salt) and 0.1-1.0 mg protein extract. The assay mixture was equilibrated at 30°C for 5 min and the reaction was initiated with the addition of 20 mM L-glutamine. The

reaction proceeded for 10 min, and was terminated by the addition of a stop mix, which contained, per litre, 55 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ; 20 g trichloroacetic acid; 21 ml 11.6 M HCl. The samples were centrifuged to remove any precipitate and the absorbance at 540 nm was recorded. The concentration of glutamyl hydroxamate produced was determined by reference to a calibration curve. The activity of the enzyme was expressed as a number of GS units, where one GS unit produces one  $\mu\text{mole } \gamma\text{-glutamyl hydroxamate per minute}$ .

The second method was to assay the forward reaction of the enzyme by the method described by Ebner et al (1970). This assay follows the formation of  $\gamma\text{-glutamyl glutamate}$  from glutamate and hydroxylamine. Assays were performed in a final volume of 500  $\mu\text{l}$  containing 94 mM imidazole-HCl pH 7.0; 47 mM hydroxylamine; 56 mM  $\text{MgCl}_2$ ; 168 mM sodium glutamate and 94  $\mu\text{g}/\mu\text{l}$  CTAB and 0.1-1.0 mg protein extract. After equilibration at 30°C, the reaction was initiated with 60  $\mu\text{l}$  0.2 M ATP pH 7.0. The reaction was incubated at 30°C for 10 min. and was terminated with 1 ml stop mix (as described for the reverse reaction). The absorbance was measured at 540 nm and the activity determined as above.

The final method was to measure the biosynthetic activity of the enzyme. This measures the release of Pi from ATP during the reaction



Assays were performed in a final volume of 500  $\mu\text{l}$  and contained 50 mM imidazole-HCl pH7.15; 100 mM sodium glutamate; 50 mM  $\text{NH}_4\text{Cl}$ ; 50 mM

MgCl<sub>2</sub>; and 0.1-1.0 mg protein extract. After equilibration at 30°C, the reaction was initiated with the addition of 60 µl ATP (0.1 M). After incubation at 30°C for 10 min, the reaction was terminated by the addition of 1 ml 10% trichloroacetic acid (TCA). The phosphate released was determined by the method described by Baginski et al (1967). 1 ml 2% (w/v) ascorbic acid in 10% TCA was added to the sample. To this was added 0.5 ml 1% (w/v) ammonium molybdate tetrahydrate. After mixing, 1 ml of a mixture containing 2.0% (w/v) sodium citrate dihydrate; 2% (w/v) anhydrous sodium arsenite and 2% (v/v) glacial acetic acid was added. The reaction was mixed and left at room temperature for 15 min. to allow the colour to develop. The absorbance at 540 nm was measured and the phosphate content determined by reference to a standard curve prepared with known amounts of phosphate.

#### 2.5.3.2 Glutamate synthase EC 2.6.1.53

Glutamate synthase (GOGAT) was assayed by the method of Meers et al (1970). Assays were performed in a final volume of 1 ml which contained, in 50 mM Tris HCl pH 7.8; 5 mM 2-oxoglutarate, 1 mM NADH and 0.1-1.0 mg protein extract. The reaction was initiated by the addition of 5 mM L-glutamine. The formation of NAD<sup>+</sup> was followed for 5 min by recording the change in absorbance at 340 nm (A<sub>340</sub>). The rate of enzyme activity was calculated as shown below:

$$\text{umoles NAD(P)H/min} = \frac{\text{change in } A_{340}}{6.2(2)}$$

(where 6.2(2) is the extinction coefficient of NAD(P)H)

#### 2.5.3.3 Glutamate dehydrogenase EC 1.4.1.3

Glutamate dehydrogenase (GDH) was assayed by following the aminating reaction of Meers et al (1970). The reaction was performed in a final volume of 1 ml and contained, in 100 mM Tris HCl pH 8.5; 160 mM ammonium chloride, 1 mM NADH and 0.1-1.0 mg protein extract. The reaction was initiated by addition of 20 mM 2-oxoglutarate. The activity was determined as shown in section 2.5.3.2.

#### 2.5.3.4 Alanine dehydrogenase EC 1.4.1.1

Alanine dehydrogenase (ADH) was assayed by the method described for GDH, except sodium pyruvate (5 mM) replaced 2-oxoglutarate.

#### 2.5.3.5 Nitrate reductase

Nitrate reductase activity was determined by following the conversion of nitrate to nitrite. The reaction was performed in a total volume of 1 ml containing, in 100 mM phosphate buffer pH 7.0; 10 mM potassium nitrate, 10 mM methyl viologen, 1.5 mM sodium dithionite, 95  $\mu$ M sodium hydrogen carbonate. The reaction was performed in glass tubes and was equilibrated at 30°C for one minute, and initiated by addition of 0.1-1.0 mg protein extract. The reaction proceeded for 5 min, and was terminated by rapid vortexing to completely oxidize the sodium dithionite. The nitrite content was determined by addition of 1 ml sulphanilamide and 1 ml N-NEDH. This was allowed to stand at room temperature for 30 min, and the absorbance at 540 nm was recorded. Nitrite content was calculated by reference to a calibration curve over the range 1-10 mM nitrite.

#### 2.5.4 Carbon Assimilation Pathway Enzymes

Cells and extracts for these enzyme assays were prepared from cells grown in ANMS media.

##### 2.5.4.1 Methane monooxygenase

Whole cells were assayed for methane monooxygenase activity by measuring the conversion of propylene to propylene oxide. Assays were performed in a final volume of 1 ml in 5 ml flasks. 0.1-1.0 mg dry weight cells were added to 20 mM phosphate buffer, pH 7.6, which contained 1.5% (w/v) sodium chloride. The flask was sealed with a rubber suba-seal stopper. 5 ml air were removed and 5 ml propylene were added. 50  $\mu$ l samples of the assay mixture were removed at 0 min and at 5 min intervals and the concentration of propylene oxide was measured on a gas chromatograph, fitted with a Poropak Q column. This was previously calibrated with a standard solution (5  $\mu$ l 1 mM stock solution) of propylene oxide. Methanol (1 mM; 5 mM; 10 mM) and sodium formate (5 mM; 10 mM; 50 mM) were added as electron donors to enhance the activity of methane monooxygenase.

##### 2.5.4.2 Methanol dehydrogenase (PMS-Linked Activity)

Methanol dehydrogenase was assayed for phenazine methosulphate (PMS) linked activity. This was measured spectrophotometrically by following the reduction of dichlorophenol indophenol (DCPIP) by reduced PMS. Assays were performed in a final volume of 1.5 ml, which contained, in 20 mM Tris NaOH pH 9.0 (previously sparged with oxygen-free nitrogen for 1 minute), 0.11  $\mu$ mol PMS, 0.13  $\mu$ mol DCPIP, 45  $\mu$ mol  $\text{NH}_4\text{Cl}$  and 0.1-1.0 mg cell-free protein extract. The cuvettes were

equilibrated at 30°C for one minute before the reaction was initiated by addition of 10  $\mu$ mol methanol. The change in absorbance at 600 nm was followed for 5 min.

#### 2.5.4.4 Hexulose phosphate synthetase

Hexulose phosphate synthetase was assayed spectrophotometrically by following the conversion of  $\text{NADP}^+$  to NADPH (Dahl *et al*, 1972). The assay was performed in a final volume of 1 ml which contained, in 100 mM phosphate buffer pH 7.0, 4 mM magnesium chloride, 0.25 mM  $\text{NADP}^+$ , 4 mM formaldehyde, 1.68  $\mu$ molar units rabbit muscle phosphoglucose isomerase, 0.15  $\mu$ molar units yeast glucose-6-phosphate dehydrogenase and 0.05-0.15 mg cell-free extract. The assay was initiated by the addition of 5 mM D-ribose-5-phosphate, and the absorbance at 340 nm was recorded over 5 min. The rate of activity was calculated as shown in section 2.5.3.2.

#### 2.5.4.5 Hydroxypyruvate reductase

Hydroxypyruvate reductase was assayed spectrophotometrically by following the conversion of NADH to  $\text{NAD}^+$  (Large and Quayle, 1963). The assay was performed in a final volume of 3 ml which contained, in 100  $\mu$ moles phosphate buffer pH 7.6, 0.4  $\mu$ mole NADH and 0.1 mg cell-free extract. The reaction was initiated by the addition of 2  $\mu$ mol lithium hydroxypyruvate. The absorbance at 340 nm was recorded over 5 min and the rate of activity was calculated as shown in 2.5.3.2.

#### 2.5.4.6 L-serine-glyoxylate aminotransferase

L-serine-glyoxylate aminotransferase was assayed

spectrophotometrically by following the conversion of NADH to NAD<sup>+</sup> (Blackmore and Quayle, 1970). The assay was performed in a final volume of 1 ml which contained, in 50  $\mu$ mol phosphate buffer pH 7.1, 0.01  $\mu$ mol pyridoxal phosphate, 0.15  $\mu$ mol NADH, and cell-free extract (up to 0.2 mg protein). The reaction was initiated by the addition of 5  $\mu$ mol sodium glyoxylate, and the absorbance at 340 nm was recorded for 2-3 min. 5  $\mu$ mol L-serine were added, and the absorbance was followed for a further 2-3 min. The rate was calculated as shown in 2.5.3.2, and the endogenous rate obtained before the addition of L-serine was subtracted from the rate obtained after the addition of L-serine.

## 2.6 DNA preparation

DNA concentration was determined by measuring the absorbance at 260 nm on a Pye Unicam SP1800 spectrophotometer. An absorbance of 1.0 was equivalent to 50  $\mu$ g.ml<sup>-1</sup> DNA.

To remove protein from DNA solutions, the samples were phenol or phenol/chloroform extracted. Phenol was prepared by distillation, and storage at -20°C until needed. The phenol was then melted at 60°C, and equilibrated with an equal volume of TE (10 mM Tris pH 8.0; 1 mM diaminoethanetetraacetic acid, EDTA)). The aqueous phase was removed and the phenol again equilibrated with TE. This was repeated until the aqueous phase remained at pH 8.0. 8-hydroxyquinoline was added at a concentration of 0.15 (w/v). Phenol/chloroform was prepared by addition of chloroform and isoamyl alcohol to distilled phenol in the ratio 25:24:1 (phenol:chloroform:isoamyl alcohol).

DNA was precipitated by addition of 1/50th volume 5 M sodium

chloride and 2 volumes ethanol, unless otherwise stated. The DNA solution was incubated at -20°C for 2 to 12 hr, and pelleted by centrifugation at 20,000 rpm, 30 min at 4°C (Beckman J2-21M/E). The pellet was dried by vacuum desiccation, and resuspended in TE.

Purification of DNA was by caesium chloride/ethidium bromide density gradient ultracentrifugation. After ethanol precipitation, DNA was resuspended in 3.8 ml TE. To this, 4.2 g caesium chloride were added. When dissolved, 1 ml (10 mg.ml<sup>-1</sup> stock) ethidium bromide was added. The DNA solution was transferred to an ultracentrifuge tube, and heat-sealed, ensuring the tube contained no air bubbles. The tube was spun at 55,000 rpm, 20°C, for 16 hr (Beckman L8 series ultracentrifuge). After centrifugation, the tube was examined under UV light and the DNA band extracted with a needle. The DNA was butanol extracted to remove ethidium bromide (Davies et al, 1980) and dialysed against 2l TE (Maniatis et al, 1982) three times.

#### 2.6.1 Plasmid DNA preparation from E. coli

Plasmid DNA was prepared from E. coli by the alkaline lysis method as described in Maniatis et al (1982).

#### 2.6.2 Chromosomal DNA preparation from methanotrophs

Chromosomal DNA was prepared from methanotrophs by a method adapted from C.J. Oakley (pers. comm.). Fermenter or batch grown cells were harvested by centrifugation and resuspended in 10 ml EDTA saline. (Cells could be stored at -20°C until needed). Cells were treated with lysozyme at 37°C until microscopic analysis revealed distortion. 3.5 ml 250 mM EDTA pH 8 were added, and the cells incubated at 37°C



for 4 min. After the addition of 0.6 ml of 35% (w/v) sarkosyl, 200  $\mu$ l proteinase K ( $10 \text{ mg.ml}^{-1}$ ) were added, and the mixture incubated at  $37^{\circ}\text{C}$  until the solution cleared. 5 M sodium perchlorate (3.5 ml) were added and the solution incubated at  $60^{\circ}\text{C}$  for 15 min. After phenol/chloroform and chloroform extraction, DNA was ethanol precipitated and pelleted by centrifugation. After resuspension in TE, DNA was caesium chloride/ethidium bromide density gradient purified.

#### 2.6.3 Chromosomal DNA preparation from *E. coli* HB101

Chromosomal DNA from *E. coli* strain HB101 was prepared as described by Marmur (1961).

#### 2.6.4 DNA preparation from environmental samples-Filtration method

Bacterial DNA was extracted from environmental samples by the method of Fuhrman et al (1988). Sea water (2-10 litres) was prefiltered under vacuum through GF/C Whatman filters to remove particulate matter and larger eukaryotic unicellular organisms. Bacteria were harvested by filtration through 0.22  $\mu$ m millipore filters and filters were stored at  $-20^{\circ}\text{C}$  until needed. The filters were then thawed, cut into strips approximately 1 cm x 2 mm and placed in conical bottomed sterile plastic universals. 4 ml STE (10 mM Tris HCl pH 8.0, 100 mM sodium chloride, 1 mM EDTA pH 8.0) were added, and the samples vortexed briefly. 1/10 volume sodium dodecyl sulphate (SDS) (10% w/v) was added dropwise with swirling, and the samples boiled for 1.25 to 2 min. The liquid was transferred to 15 ml centrifuge tubes and the filters rinsed with 1 ml STE. Cell debris was pelleted by centrifugation at 10,000 rpm,  $15^{\circ}\text{C}$ , 10 min. The supernatant was ethanol

precipitated by the addition of 1.5 ml ammonium acetate (10.5 M) and 14 ml ice-cold ethanol. The DNA was incubated at -20°C for 2 hr and pelleted by centrifugation at 20,000 rpm, 4°C, 20 min. The DNA was resuspended in 500 µl TE. After phenol extraction, phenol/chloroform extraction and chloroform extraction, DNA was reprecipitated by addition of 0.12 ml ammonium acetate (10.5 M) and 1 ml ice cold ethanol. The DNA was incubated for 1 hr at -20°C. It was then pelleted, dried under vacuum and resuspended in 300 µl TE.

#### 2.6.5 DNA preparation from environmental samples-Centrifugation method

DNA was prepared from water samples by a method of centrifugation, as described by Atlas and Bej (1990). After centrifugation at 10,000 x g, cells were resuspended in 50 µl lysis buffer (containing 1 x polymerase chain reaction (PCR) buffer, 0.05 mg.ml<sup>-1</sup> proteinase K, 20 mM dithiothreitol and 1.8 µM sodium dodecyl sulphate) (10 x PCR buffer contains 50 mM potassium chloride, 100 mM Tris HCl pH 8.13, 15 mM magnesium chloride and 0.1% (w/v) gelatin). The cells were vortexed for 15 seconds and incubated at 37°C for 1 to 1.5 hr, after which they were heated to 80°C for 5 min to inactivate the proteinase K.

#### 2.7 % Guanine and Cytosine (%G+C) content of Isolates

The %G+C content of the isolates was determined by the melting temperature method (Mandel & Marmur, 1968). DNA was prepared from the isolates as described in 2.6.2. This was dialysed overnight at 4°C against 0.5% SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), and diluted to give a concentration of approximately 20 µg.ml<sup>-1</sup>. 1.5ml

quartz cuvettes with lids were cleaned with 1 M HCl to remove all protein traces and rinsed in distilled, deionised water. 1ml DNA sample was placed into the cuvettes, and the lid sealed in place with Parafilm to prevent evaporation of the sample. The absorbance at 260nm was measured with a Cecil CE6700 spectrophotometer fitted with a thermoregulator capable of heating the unit to 110°C. The absorbance was recorded at 25°C ( $A_{25}$ ), at 50°C and thereafter at 5°C intervals until the absorbance started to rise rapidly. At this point the sample was heated at 2°C intervals and the absorbance recorded ( $A_T$ ) until no further increase in absorbance was seen. The melting temperature curve was plotted with  $A_T/A_{25}$  against temperature. The temperature at the half point of  $A_T/A_{25}$  was calculated ( $T_m$ ). %G+C content was determined by the following equation:

$$\%G+C = (T_m - 69.3) \times 2.44$$

The melting temperature curve was repeated three times for each isolate, and two times for an E. coli strain HB101 control.

## 2.8 DNA Analysis

### 2.8.1 Restriction Enzyme Digestion

Purified DNA obtained as described in section 2.6 was analysed by restriction enzyme digestion. The appropriate restriction enzyme was added to the DNA at a concentration of approximately 10 units for 1-2  $\mu$ g DNA. Commercial restriction buffer was obtained for each enzyme and used at the manufacturers recommended concentration. The digestions were incubated at 37°C for 2-3 hr. Restriction enzymes were

obtained from Bethesda Research Labs, Cambridge, England, and Amersham.

### 2.8.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise and to isolate DNA. Agarose gels were made and run in Tris-borate buffer (TBE) containing, in 1 litre, 11 g Tris base, 5.5 g boric acid and 2 ml of 50 mM EDTA, pH 8.0. Agarose (Sigma Type II, medium EEO) was added to a final concentration of 1% (w/v). Two sizes of gel were routinely used; 'minigels' (Flowgen Instruments Ltd, Sittingbourne, England) which were for preliminary screening of DNA, and 20x25 cm gels (Bethesda Research Labs, Cambridge, England), which were used for analysis of the DNA. Ethidium bromide was added either directly to the gel before casting (minigels) ( $0.5 \mu\text{g} \cdot \text{ml}^{-1}$  final volume) or after the gel had run by soaking the gel in a bath containing  $50 \text{ mg} \cdot \text{l}^{-1}$  ethidium bromide. Minigels were run at a constant current of 40 mA for 2-3 hr, large gels were run for 16 hr at a constant voltage of 50 V. DNA was visualised by placing the gel on a UV light box. Gels were photographed using a Polaroid camera (CU5 Land Camera, loaded with Polaroid 665 positive/negative film).

### 2.8.3 Elution of DNA Fragments from Agarose Gels

DNA fragments separated by electrophoresis were removed from the gel by excision of the agarose containing the fragment. DNA was eluted from agarose using a commercially available kit 'Geneclean II' (Bio 101, California, USA). The kit was used according to the manufacturer's instructions.

#### 2.8.4 Southern Blot Hybridization

Southern Blot hybridization was carried out essentially as described in Maniatis et al (1982). After hybridization, the filters were washed initially in 2 x SSC at 40°C for 30 min and exposed with an intensifying screen to X-ray film (Kodak) at -70°C for 12-96 hr. Further washes of filters at higher stringencies were performed at higher temperatures and lower concentrations of SSC as stated in the text.

#### 2.8.5 Dot Blot Hybridization

Dot Blots were prepared by using a dot blot manifold. Nitrocellulose was prewetted with 10 x SSC. and placed onto water wetted Whatman 3MM filter paper. The dot blot apparatus was set up as described by the manufacturers. DNA was denatured by heating at 95°C for 5 min and snap-cooled on ice. An equal volume of 20 x SSC was added to the denatured DNA. The DNA was then placed onto the filter and sucked onto the filter under vacuum. After the DNA was sucked onto the filter, it was soaked in denaturing solution (Southern blot denaturation solution, Maniatis et al, 1982) for 5 min, and neutralizing solution (Southern blot neutralization solution, Maniatis et al, 1982) for 1 min. The filter was then dried at 80°C in a vacuum oven, for 2 hr.

#### 2.8.6 Nick Translation of DNA fragments

Nick translation of DNA fragments was essentially as described by Maniatis (1982). 0.25 µg DNA were labelled by addition of 3 µl 'cold' nucleotide mix (containing all the nucleotides except the radio

labelled nucleotide); 1  $\mu$ l  $^{32}$ P-labelled nucleotide (dCTP or dGTP); 4  $\mu$ l '10' x nick translation buffer (NTB); 1  $\mu$ l DNA polymerase I; 1  $\mu$ l DNase I (diluted 1 in 50,000). The nick translation was incubated at 14°C for 4-6 hr. Unbound labelled nucleotide was removed from the mixture by separation of the DNA fragment on a Sephadex-G25 column. The fractions were collected and radioactivity measured by a Geiger counter.

## 2.9 Polymerase Chain Reaction (PCR)

### 2.9.1 Design of Primers

The design of primers for amplification of soluble methane monooxygenase and methanol dehydrogenase genes is described in chapter 4.

### 2.9.2 Conditions used for amplification

DNA from pure methylotroph cultures prepared as in section 2.6.2 was diluted to a concentration of  $\text{ng.ml}^{-1}$  unless otherwise stated. DNA prepared from environmental samples was used without dilution. Each amplification reaction was performed in 50  $\mu$ l, which contained, unless otherwise stated, 0.5  $\mu$ l DNA template, 0.5  $\mu$ l each primer (to give a final concentration of 0.25 mM), and 40  $\mu$ l reaction mix. The reaction mix contained, in 220  $\mu$ l, 27.5  $\mu$ l Taq polymerase buffer, supplied by the manufacturers, 5.5  $\mu$ l each nucleotide and 1.38  $\mu$ l Taq polymerase. Nucleotides, Taq polymerase and buffer were supplied by Perkin Elmer Cetus, unless otherwise stated, and used as recommended by the manufacturers. Amplification reactions were performed in sterile, siliconised Eppendorf tubes. Dilution of samples and mixes was by sterile distilled, deionised water. Control DNA template and

primers were also obtained from Perkin Elmer Cetus. Each reaction was set up, and spun briefly in a microfuge to collect droplets from the sides of the tube. 50 $\mu$ l paraffin were added to each tube to prevent evaporation.

## 2.10 Sequencing DNA

### 2.10.1 Preparation of template

PCR products were sequenced by an adaptation of a method published by Pharmacia (1991). PCR products were cleaned by 'Gene-clean' as described by the manufacturers to remove excess nucleotides, Taq polymerase and paraffin oil. The DNA was resuspended in 20  $\mu$ l of TE. Approximately 0.5  $\mu$ g PCR product in a total volume of 8  $\mu$ l TE was denatured by the addition of 1  $\mu$ l 1 M NaOH. After a brief vortex, this was incubated at room temperature for 10 min. The template was neutralized by the addition of 1  $\mu$ l 1 M HCl, then 2  $\mu$ l PCR primer were added immediately. The primer was added to give a primer:template ratio of 5:1. This was followed by the addition of 2 $\mu$ l 5 x buffer (1 x buffer contains 40mM Tris HCl pH 7, 50mM sodium chloride and 100mM magnesium chloride). The reaction was incubated at 37°C for 15 minutes to anneal the primer to the template. Labelling mix containing (in 5.5  $\mu$ l final volume) 1  $\mu$ l 0.1 M dithiothreitol; 1.5  $\mu$ M each dCTP, dTTP and dGTP, 5  $\mu$ Ci  $^{35}$ S-dATP and 0.2 $\mu$ l 'Sequenase' enzyme (United States Biochemicals, Cleveland, USA) was added to each tube, mixed and left at room temperature for 5 min. Into one of each of four wells of a microtitre plate, 2.5 $\mu$ l termination mix for each of the four nucleotides were added. These contained 80 $\mu$ M each dATP, dCTP, dGTP, and dTTP; 8 $\mu$ M ddNTP, (where ddNTP is ddATP, ddCTP, ddGTP or ddTTP) and

50mM sodium chloride. To each well, 3.6µl labelled template mix were added. The microtitre plate was spun briefly in a plate centrifuge and incubated at 37°C for 10 min. To each well, 2µl formamide dye (containing 100ml formamide deionised with mixed bed resin, 0.1g xylene cyanol; 0.1g bromophenol blue and 2ml 0.5M EDTA pH 8.0) were added and incubated at 80°C for 15 min. Labelled sequencing products were then separated by polyacrylamide gel electrophoresis.

### 2.10.2 Polyacrylamide Sequencing Gels

Sequencing reaction products were separated on wedged polyacrylamide gels using a "Base Runner" nucleic acid sequencer (International Biotechnological Incorporated, Connecticut, USA). 100 ml Acrylamide solution (containing, in 500 ml, 1 x Tris borate buffer, 230 g urea and 40% 19:1 acrylamide:bis-acrylamide solution) was solidified with 40 µl N N N'N'-tetramethyl-1,2-diaminoethane and 667 µl ammonium persulphate (0.1 mg.ml<sup>-1</sup>). After the gel was poured and set, it was pre-run for 30 min at 55 W and the samples loaded. The sequencing reaction products were separated on the gel for 2½ hr at 55 W. The gel was fixed in 10% methanol/10% acetic acid for 30 min and dried on a vacuum gel drier at 80°C for 3 hr. The gel was exposed to Fuji X-ray film for between 12 and 72 hr.

### 2.11 Whole cell antibodies

#### 2.11.1 Raising whole cell antibodies

Antibodies to marine methanotroph isolates were raised in rabbits (Tan Lop rabbits). 50 ml cultures of the isolates, at an absorbance at 540 nm of 1.0, were harvested by centrifugation at 18,000 rpm for



20 min. The cell pellet was resuspended in physiologically buffered saline (PBS) and reharvested. The pellet was resuspended in 1 ml PBS, and 0.5 ml inoculated into the rabbit intravenously. Approximately 5 ml blood was removed at the time of inoculation (pre-immune serum), allowed to clot at 4°C overnight, and the cells separated from the serum by centrifugation. The serum was stored at -20°C until needed. A further 5 ml blood was removed from the rabbit after 5 weeks. Six weeks after the initial inoculation, the rabbit was reinoculated as before, and blood removed after 2 weeks.

#### 2.11.2 Detection of antibodies by passive haemagglutination

Antigen was prepared by saline extraction: 50 ml culture of antigenic culture was harvested by centrifugation and resuspended in 4 ml 0.1 M saline. The culture was then incubated at 100°C for 1 hr, and was centrifuged 18,000 rpm 10 min. The supernatant contained the antigen. The antigen was then diluted 1:10 with PBS and incubated for 1 hr at 37°C with an equal volume 1% sheep red blood cells (SRBC) (in PBS). The antigen was centrifuged and washed 3 times in PBS, then resuspended to give a final volume of 0.5% SRBC.

Antisera were diluted 1:40 in PBS, and 2-fold dilutions were made of these. 25 µl each dilution were placed into the wells of a microtitre plate, together with pre-immune sera and PBS containing no antisera. 25 µl antigen/SRBC were added to each well, and the plates incubated at 37°C for 1 hr. Plates were then stored overnight at 4°C, and examined for agglutination. The highest dilution of antisera that showed agglutination indicated the titre of the antiserum.

## 2.12 Environmental Sampling

Environmental measurements were taken from sites in two areas of the Southern ocean, and are shown in fig. 5.1 and 5.2. Water was sampled through the water column and was collected by a CTD sampler (conductivity, temperature, depth). The CTD used contained 10 bottles with a volume of 2.5 l each, placed around a frame. Attached to the frame were sensors to detect conductivity, temperature, depth, and light intensity. The CTD was connected to a computer situated on the ship and through this, information was passed to and from the CTD. The bottles around the CTD could be closed at the required depth, trapping water from that depth for analysis. A picture of the CTD used is shown in fig. 2.1.

### 2.12.1 Methane Concentration Measurements

Methane concentration in water samples were measured by a 'head-space' equilibrium technique. Water from different depths was removed from the CTD bottles before exposure to air. 5 ml samples were introduced to sealed nitrogen flushed tubes by a needle and syringe. The tubes were shaken vigorously for 30 seconds and incubated at 25°C for 2-3 hr with vigorous shaking every 30 min. A 250  $\mu$ l sample of gas from the head space was withdrawn with a Hamilton gas-tight syringe, and introduced into a gas chromatograph. The conditions set on the gas chromatograph for methane measurement were determined on board and are described in chapter 5. The concentration of methane in each sample was calculated by comparison with standard samples as described in chapter 5. The measurements were performed in triplicate for each depth.

### 2.12.2 Methane Oxidation Rates

The rate of methane oxidation in water samples was calculated by a method based on Ward *et al.* (1967). 25-ml plastic syringes

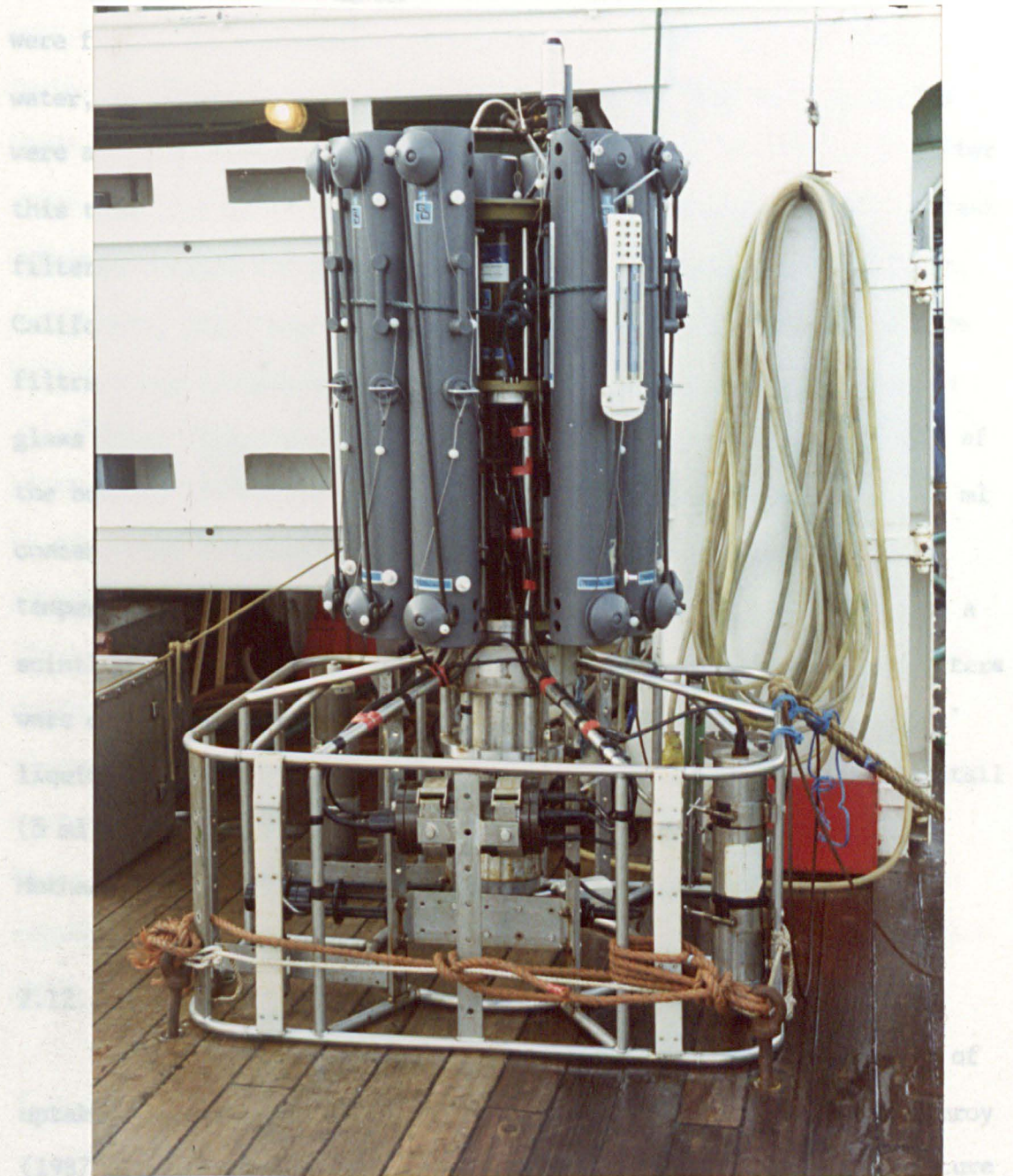


Fig. 2.1 : CTD sampler used for water collection in the Southern Ocean.

### 2.12.2 Methane Oxidation Rates

The rate of methane oxidation in water samples was calculated by a method based on Ward et al (1987). 250ml plastic conical flasks were filled and stoppered with rubber 'suba-seal' stoppers under water, to ensure removal of all air. 100  $\mu$ l of  $^{14}\text{C}$ -labelled methane were added by syringe, and the flask incubated at 4°C for 24 hr. After this time, 0.1 ml of 10M NaOH were added and the contents of the flask filtered through a 0.22 $\mu$ m Nucleopore filter (Nucleopore corporation, California, USA). The filter was stored in a scintillation vial. The filtrate was transferred to a 300 ml glass bottle, which contained a glass fibre filter soaked in phenethylamine suspended from the top of the bottle. The bottle was sealed with a Suba-seal stopper and 1.0 ml concentrated sulphuric acid was added. This was incubated at room temperature for 1 hr and the glass fibre filter was also stored in a scintillation vial. The vials were stored in a desiccator. The filters were counted on a liquid scintillation counter (LKB 1212 'minibeta' liquid scintillation counter) after addition of scintillation cocktail (5 ml 'Optiphase safe', Pharmacia) on return to the laboratory. Methane oxidation measurements were performed in triplicate.

### 2.12.3 Bacterial Activity Measurements

Bacterial activity measurements were made by the measurement of uptake of tritiated thymidine ( $^3\text{TdR}$ ) as described by Joint and Pomroy (1987). 10 ml samples were placed into sterile plastic tissue culture tubes.  $^3\text{TdR}$  was added to each tube to give a concentration of 5 nmol.l $^{-1}$ . As a control, 100  $\mu$ l glutaraldehyde (electron microscope grade) was added to one of the five replicate tubes taken from each

depth immediately after the addition of the  $^3\text{TdR}$ . The samples were incubated at  $4^\circ\text{C}$  for 1 hr. Ice-cold trichloroacetic acid (TCA) was then added to give a final concentration of 5% (v/v). The samples were left at  $4^\circ\text{C}$  for 15 min, and then filtered through 25 mm  $0.2\ \mu\text{m}$  Nucleopore filters. Each filter was rinsed twice with 5 ml ice cold 5% (w/v) TCA, and the filters were then placed in scintillation vials and dried in a desiccator. Filters were counted on a liquid scintillation counter on return to the laboratory.

#### 2.12.4 Total bacterial counts

Samples for enumeration of bacteria were fixed with 2.5% (v/v) glutaraldehyde (electron microscope grade) and stored in clean bottles at  $4^\circ\text{C}$  until counted. Samples were stained with 4'6-diamidino-2-phenylindole (DAPI) (200  $\mu\text{l}$  of a  $0.1\ \mu\text{g}.\text{ml}^{-1}$  stock in 2 ml sample) (Porter & Feig, 1980) and fluorescent bacteria were counted with an epifluorescent microscope by the method of Hobbie et al (1977). The microscope used was a Leitz Ortholux II equipped with 50 W HBO light source, Ploempak 2.2 fluorescence vertical illuminator with filter block A, and an NPL Fluorotar 100/1.32 oil objective lens. For each sample, 15 to 20 fields of view were counted, giving a minimum of 300 cells sample $^{-1}$ .

#### 2.13 Photography

Photography of DNA agarose gels was performed with a Polaroid Land Camera CU5, as described in section 2.8.2. Autoradiograph x-ray film was developed in Kodak LX24 (diluted 1 part developer to 4 parts water) for 2 min, and fixed in Kodak Unifix (diluted 1 part fixer to 3

parts water) for 5 min. All other photography was performed using a Minolta 35mm camera loaded with Kodak T-max 100 film. Negatives were developed in Ilford contrast FF (diluted 1 part developer to 4 parts water) for 2 min and fixed in Kodak Unifix (diluted 1 part fixer to 3 parts water) for 5 min. Negatives, including Polaroid instant negatives, were printed onto Kodachrome II RC F3M photography paper and developed in Ilford contrast FF (diluted 1 part developer to 9 parts water) for 30 sec and fixed in Kodak Unifix (diluted 1 part fixer to 8 parts water) for 5 min.

ISOLATION AND CHARACTERIZATION OF  
MARINE METHANOTROPHS

### 3.1 Introduction

Whilst methanotrophs from terrestrial and fresh water habitats have been studied in some detail (as reviewed by Green, 1991), there appears to be little information on the isolation of methanotrophs from the marine environment. The few reports of marine isolates in the literature (Sieburth et al, 1987; Lidstrom et al, 1988) give little detail on the characteristics of those organisms.

There have been several reports that marine bacteria are hard to culture by conventional laboratory methods. Some bacteria do not grow readily on solid media. This was demonstrated by the marine methanotrophs isolated by Sieburth et al (1987) which grew poorly on solid media and growth in liquid media was also slow.

The enrichment of methanotrophs, from a range of marine habitats, using several methods was therefore considered essential. For characterization studies, new strains needed to be isolated. As there have been so few methylotrophs isolated from marine habitats, attempts have also been made to enrich and isolate methanol and trimethylamine utilizing bacteria.

Two newly isolated methanotrophs were characterized using several approaches. Firstly, the general characteristics were investigated as an aid to classification and to compare the new isolates with previously isolated marine strains. Secondly, the growth characteristics of the two isolates were examined as an aid to understanding the growth requirements of marine methanotrophic bacteria. Thirdly,



enzyme pathways and the activities of key enzymes were examined. The enzymes selected for study were enzymes of particular interest in other methanotrophs and were therefore chosen to aid comparison between the newly isolated marine strains and well characterized culture collection strains isolated from terrestrial and fresh water environments.

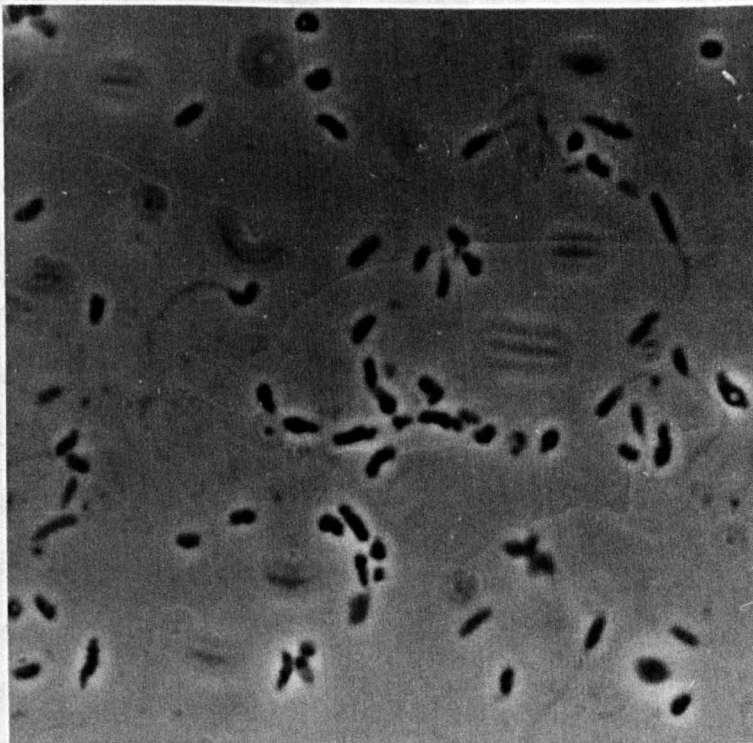
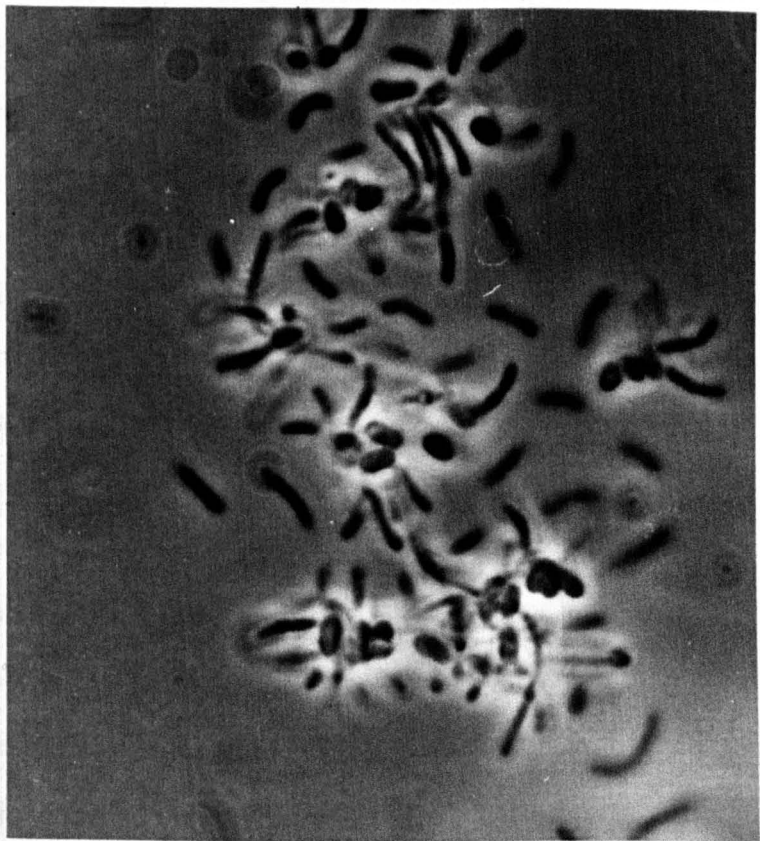
### 3.2 Methylotroph enrichments

Sea water samples from several sites around Plymouth Sound and from sites in other sea water areas were used to obtain enrichments of methylotrophs. Fresh water samples were also used to obtain methanotroph enrichment cultures to compare the detection techniques discussed in chapter 4 with conventional cultivation of methanotrophs. Areas sampled are detailed in table 3.1. Several bacterial types were obtained from different environments, although it was noted that two distinctive morphological types of methanotrophic bacteria were present in most samples after enrichment on methane. The presence of these bacteria in enrichment cultures does not necessarily reflect their dominance in the samples tested, but instead may indicate that they are the bacteria most adapted to growth in the conditions used for selection. The variety of bacterial types found in each enrichment culture is shown in fig. 3.1.

Methanol- and trimethylamine- utilizing bacteria were obtained from filter and flask enrichments in all areas tested. Many of the colonies obtained on filters incubated on sea water solidified with Noble agar were pigmented. None of the methanol or trimethylamine

AREA SAMPLED	ENRICHMENT TECHNIQUE
<i>Inshore (P.S)</i>	<i>flask</i>
<i>"</i>	<i>filter</i>
<i>"</i>	<i>fermenter</i>
<i>Deep offshore (P.S)</i>	<i>bottle</i>
<i>"</i>	<i>flask</i>
<i>"</i>	<i>filter</i>
<i>"</i>	<i>fermenter</i>
<i>Shallow offshore (P.S)</i>	<i>flask</i>
<i>"</i>	<i>filter</i>
<i>Estuary</i>	<i>flask</i>
<i>"</i>	<i>filter</i>
<i>Mediterranean*</i>	<i>flask (2 l)</i>
<i>Newbold Comyn Leamington*</i>	<i>flask (2 l)</i>
<i>N.A.C. Stoneleigh*</i>	<i>flask (2 l)</i>
<i>River Leam*</i>	<i>flask (2 l)</i>
<i>Grand Union Canal*</i>	<i>flask (2 l)</i>
<i>Tocil Lake Coventry*</i>	<i>flask (2 l)</i>
<i>East Site Pond Coventry*</i>	<i>flask (2 l)</i>

**Table 3.1: Sites sampled for methylotroph enrichment with the techniques used at each site. Areas around Plymouth Sound are shown by (P.S). The sites marked with \* were enriched for methanotrophs to confirm results of detection by molecular techniques (see chapter 4).**



***Fig. 3.1:*** Light microscope photographs of methanotroph enrichment cultures. Water samples collected from areas around Plymouth Sound.

enrichment cultures showed any growth when subcultured into liquid media and incubated with methane as a sole carbon source. Cultures initially enriched in methanol media, however, showed subsequent growth on methylamine. Similarly, cultures initially enriched in methylamine media showed subsequent growth on methanol.

The different methods of enrichment were not found to be equally effective in selecting methanotrophic bacteria. In general, bottle and fermenter enrichment procedures were found to be most effective in promoting methanotrophic growth, although these methods gave rise to fewer distinct bacterial types. Filter enrichment procedures encouraged the growth of a wider variety of distinct morphological types. Initial flask enrichment cultures where 50 ml sea water samples were added to 250 ml flasks gave no growth of methanotrophs. However, subsequent flask enrichments, where a 1 l water sample was added to a 2 l flask, gave rise to rapid growth of turbid cultures of methanotrophs. This method of enrichment was used routinely to check for the presence or absence of methanotrophs in a water sample as a comparison with the molecular detection methods described in section 4. Bottle enrichments also proved to be a reliable method of obtaining methanotrophic cultures from sea water samples. Sieburth et al (1987) reported that the addition of formaldehyde to bottle enrichments enhanced the enrichment of methanotrophs from marine samples. This observation could not be repeated with the samples used in this study. This is not surprising as formaldehyde is toxic to most bacteria, including methanotrophs. Methanotrophs were obtained from filter enrichments when the filters were placed into flasks, but no methanotrophic colonies were observed on filters placed onto solidified sea

water or NaCl/ANMS media. Inoculants taken from turbid methanotrophic cultures obtained from liquid enrichment also showed no growth when spread onto solid media. The growth of isolates on media solidified by a variety of agents was subsequently investigated and the results presented in section 3.5. It was apparent that the most successful methods for enriching methanotrophic bacteria were those in which the oxygen level was lower than atmospheric levels. This led to an investigation of microaerophilic growth of isolates and the results are presented in section 3.4.

All enrichment cultures were subcultured at least twice into fresh media. In addition, growth of methanotrophs was checked by inoculating carbon-free media and Luria broth media (containing 1.5% sodium chloride) with aliquots from the enrichment cultures. No growth was apparent in either media, which confirmed that the bacterial cultures required methane for growth and that they could not utilize complex carbon sources, as is the case for all methanotrophs so far described. The bacteria described in table 3.2 persisted through subculturing. Two of the bacteria in the fermenter enrichments were chosen for further characterization studies. These bacteria both showed rapid growth in liquid culture and both grew to a relatively high cell density (see section 3.12 for further information on growth conditions of these isolates). Both enrichment cultures contained the short fat rod shape that was typical of methanotrophic bacteria in all methane enrichments. These bacteria have been named IR1 and DR1.

<b>FLASK ENRICHMENTS</b>	<i>(inshore water)</i>				
Methylamine	pigmented colonies, mainly rods				
Methanol	rods + vibrios				
Methane	no turbidity observed				
<b>BOTTLE ENRICHMENTS</b>	<i>(offshore deep and surface water)</i>				
Methane					
+ formaldehyde	no turbidity observed				
- formaldehyde	large + small rods				
<b>FILTER ENRICHMENTS</b>	<i>(inshore/offshore deep and surface/estuary)</i>				
Methylamine	{ growth on plates and in flasks -many different morphologies				
Methanol					
Methane	<table> <tr> <td><i>plates</i></td><td><i>flasks</i></td></tr> <tr> <td>no growth</td><td>rods/cocci/pear-shaped organisms</td></tr> </table>	<i>plates</i>	<i>flasks</i>	no growth	rods/cocci/pear-shaped organisms
<i>plates</i>	<i>flasks</i>				
no growth	rods/cocci/pear-shaped organisms				
<b>FERMENTER ENRICHMENTS</b>	<i>(inshore and offshore deep water)</i>				
	large + small rods				

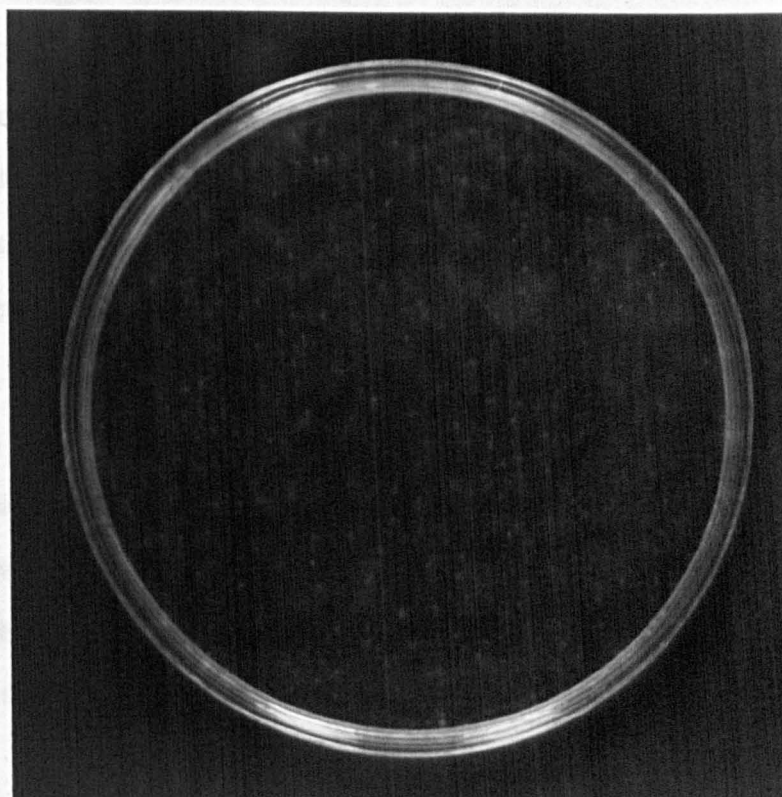
**Table 3.2 : Growth of methylotroph enrichment cultures from marine water samples. Bacteria persisted through two transfers and growth was due to utilization of C<sub>1</sub> compound.**

### 3.3 Isolation of single colonies of IR1 and DR1

Initial subculturing of IR1 and DR1 enrichments was carried out in sterile sea water. ANMS media (Whittenbury et al, 1970) with the addition of 1.5% sodium chloride (NaCl/ANMS), phosphate and vitamin supplements was found to be a suitable artificial substitute and was used routinely as a defined minimal medium.

As described above, enrichment cultures could not be sub-cultured onto solid media. Single colonies of IR1 and DR1 were therefore obtained by preparing pour plates as described in section 2.4. The plates were incubated at 30°C, with great care taken to avoid movement. After five days, individual colonies were visible with the naked eye (see fig 3.2). Colonies which were clearly isolated were sub-cultured into 5 ml media using a Pasteur pipette. After incubation with methane for three days, the cultures were turbid. The cultures were used to prepare a dilution series and the procedure was performed again. After a third dilution series was plated and colonies isolated, pure cultures of IR1 and DR1 were obtained. The purity of the cultures was confirmed by light microscopy. This procedure was also performed at intervals when stock cultures of IR1 or DR1 appeared contaminated after microscopic examination.

Cells of both IR1 and DR1 in pure culture showed extensive clumping when stored at 4°C. Stocks were therefore subcultured weekly and stored at room temperature after initial growth.



**Fig. 3.2 'Sloppy agar plate showing colonies of IR1. Colonies were isolated with a Pasteur pipette and inoculated into liquid media.**

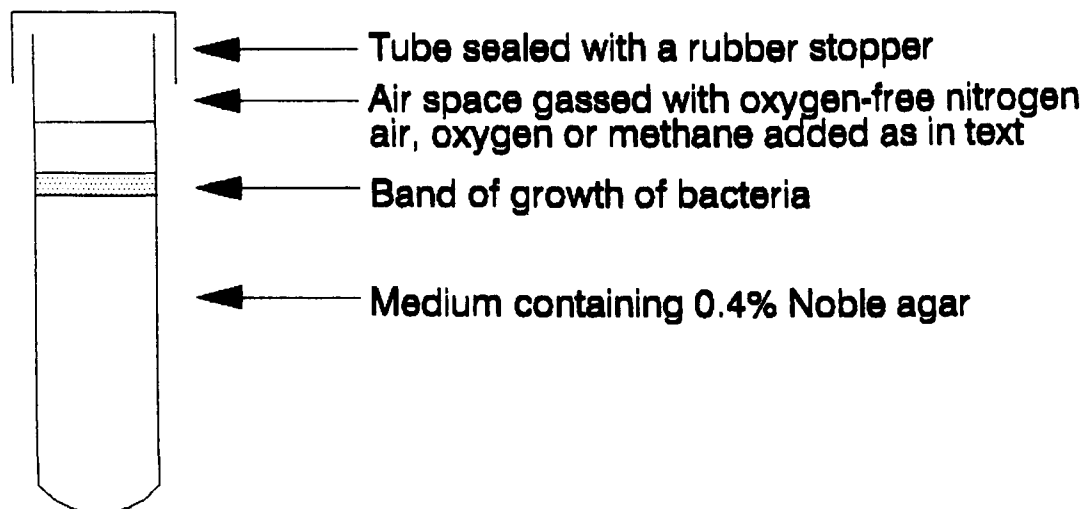


### 3.4 Investigation of microaerophilic growth

Microaerophilic growth was investigated for IR1 and DR1 after it was observed that the initial enrichment cultures showed rapid growth in lowered oxygen levels in fermenter and bottle enrichments. Sloppy agar tubes were set up as described in section 2.4 and incubated at 30°C. After five days, the tubes were examined. Growth of the isolates appeared as a band in the agar (see fig 3.3). The conditions tested and the results obtained are shown in table 3.3. The bands of growth obtained for both isolates was observed to be slightly lower in tubes containing high concentrations of oxygen. This indicated that IR1 and DR1 showed oxygen sensitivity, but as there was little difference between the tubes, it is unlikely that this is true microaerophily. Tubes set up to investigate the nitrogen-fixing ability of the isolates showed no growth. This indicates that IR1 and DR1 cannot fix nitrogen (see section 3.10).

### 3.5 Growth of IR1 and DR1 on solid media

NaCl/ANMS and sea water were solidified with a variety of different agents to test the growth of IR1 and DR1. The solidifying agents tested are listed in table 2.1 and included agars, agarose, silica and specialized compounds such as Ludox. Growth was not observed on any of the solid media tested. This was initially thought to be due to a toxic effect of agar or agarose, but non-agar based agents (e.g. Polyol F127, silica plates and Ludox) also inhibited growth. Traditional methods of methanotroph isolation (i.e. liquid enrichments and then selection on solid media (for example, Whittenbury et al, 1970a)) therefore underestimates the number and diversity



***Fig. 3.3:*** Microaerophilic growth of isolates IR1 and DR1. appearance of tubes after incubation at 30° C for five days. See text for further details.

	<b>FLUSHED</b>	<b>METHANE</b>	<b>AIR</b>	<b>GROWTH</b>
1+2	no	yes	no	yes
3+4	N <sub>2</sub>	yes	no	no
5+6	N <sub>2</sub>	yes	yes	yes
7+8	O <sub>2</sub>	yes	no	yes
9+10	O <sub>2</sub>	no	no	no
11+12	N <sub>2</sub>	no	no	no
13+14	no	no	no	no
15+16*	no	no	no	no
17+18*	N <sub>2</sub>	yes	yes	no
19+20*	N <sub>2</sub>	no	no	no

**Table 3.3: Results of investigation into microaerophilic growth. The conditions shown above were repeated in duplicate for both IR1 and DR1. Growth of IR1 and DR1 was observed in tubes 1+2; 5+6 and 7+8, when methane and air were both present. Tubes 3+4 showed no growth, illustrating that IR1 and DR1 both require O<sub>2</sub> for growth. No growth is observed in tubes lacking methane (9+10; 11+12 and 13+14). IR1 and DR1 in tubes 7+8 gave a band of growth which was slightly lower than that seen in tubes 1+2 and 5+6. IR1 and DR1 therefore show a sensitivity to the higher oxygen levels found in tubes 7+8, but this is not due to true microaerophily. Tubes marked with an asterisk were inoculated with IR1 and DR1 in nitrogen-free media to investigate the nitrogen fixing ability of IR1 and DR1. There was no growth observed in any of these tubes.**

of methanotrophs present in the environment. Growth was obtained, however, on a semi-solid medium containing 0.4% Noble agar added to both NaCl/ANMS and sea water. The use of this medium in isolating pure cultures of IR1 and DR1 is described above.

### 3.6 Growth on low concentrations of iron

Methanotrophs often produce a pigment when grown under "low iron" conditions (Green, 1991). 0.4% Noble agar/NaCl/ANMS plates were made with a reduced iron content as described in Section 2.4. After growth the plates were examined, IR1 and DR1 produced no such pigment.

### 3.7 Antibiotic resistance profiles

The antibiotic resistance profiles of IR1 and DR1 were investigated. A range of antibiotics were added to liquid media and were also added as discs on 0.4% Noble agar/NaCl/ANMS plates. After incubation, the presence or absence of growth was observed. On plates, the zone of inhibition was measured. The resistance of IR1 and DR1 to the antibiotics tested is shown in table 3.4.

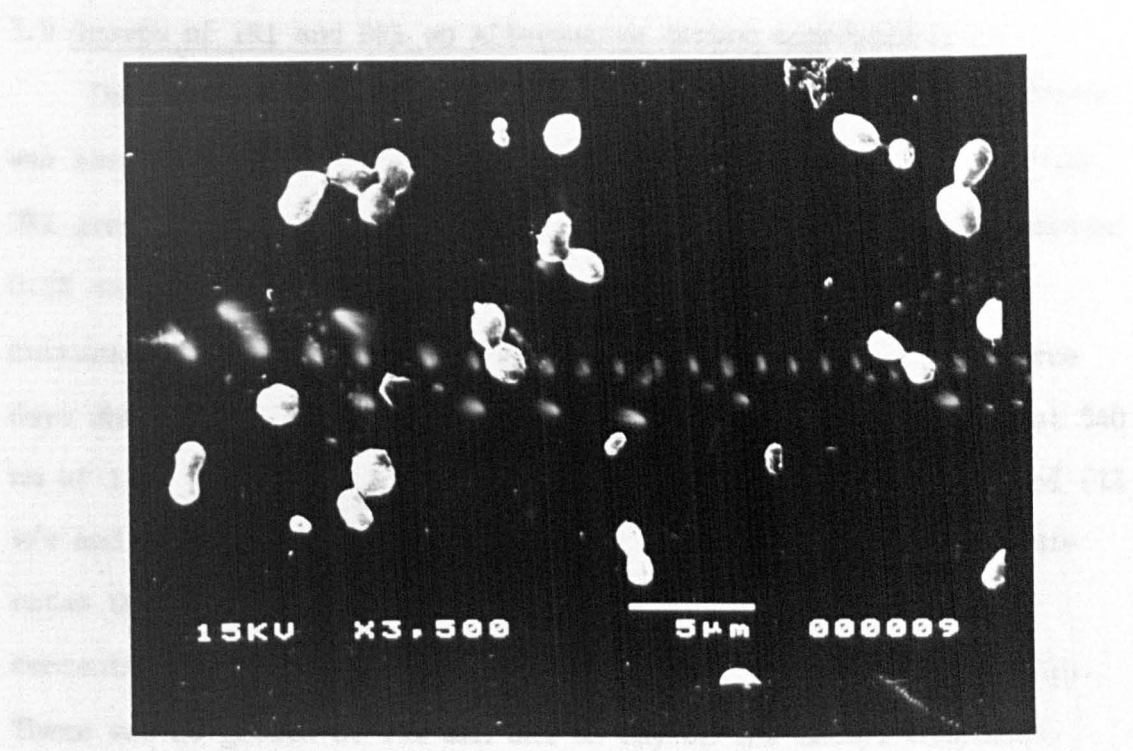
### 3.8 Physical characteristics of IR1 and DR1

IR1 and DR1 were Gram negative, motile, short fat rods with dimensions of  $1.4 \times 0.8 \mu\text{m}$  for IR1 and  $1.3 \times 0.8 \mu\text{m}$  for DR1 . Scanning electron microscopy was performed on IR1 and the micrograph shown in fig. 3.4. Both isolates appeared white/buff in liquid culture. Colonies on sloppy agar plates were opaque white. Centrifugation of liquid cultures gave an orange/pink pellet. Both isolates appeared to possess a capsule after Hiss' capsule stain and Indian ink staining.

ANTIBIOTIC	IR1	DR1
Kanamycin (25 $\mu\text{g ml}^{-1}$ )	S	S
Streptomycin (10 $\mu\text{g ml}^{-1}$ )	R	R
Trimethoprim (10 $\mu\text{g ml}^{-1}$ )	R	R
Ampicillin (12.5 $\mu\text{g ml}^{-1}$ )	S	S
Tetracyclin (10 $\mu\text{g ml}^{-1}$ )	S	S
Chloramphenicol (10 $\mu\text{g ml}^{-1}$ )	S	S
Gentamycin (10 $\mu\text{g ml}^{-1}$ )	S	S
Penicillin (1 unit)	R	S

**Table 3.4: Antibiotic resistance profiles of isolates IR1 and DR1. Resistance to the antibiotics listed above is represented by R; sensitivity by S. Both isolates showed resistance to streptomycin and trimethoprim but only IR1 was resistant to penicillin G.**

IR1 and IR2 showed positive for urease and indole. The isolates were viable and the presence of flagella was verified by phase contrast. This was confirmed by transmission electron microscopy. The cells appeared to possess either a single polar flagellum or two flagella. Flagella clumped at one end. Further characterisation and physiological characteristics are summarised in table 3.1.



Isolates IR1 and IR2 also showed no growth on minimal medium or yeast extract. This indicates that both IR1 and IR2 have very strict nutritional requirements for nitrogen and, in common with most validated isolates from this source, lack the ability to utilise any other carbon compound except urea.

**Fig. 3.4 : Scanning electron micrograph of isolate IR1.**

IR1 and DR1 tested positive for catalase and oxidase. The isolates were mobile and the presence of flagella was tested by Grays method. This was confirmed by transmission electron microscopy. The isolates appeared to possess either a single polar flagellum or several long flagella clumped at one end. Rosette formation was not evident. The characteristics are summarised in table 3.5.

### 3.9 Growth of IR1 and DR1 on alternative carbon compounds

The growth of IR1 and DR1 on carbon compounds other than methane was investigated and is shown in table 3.6. Besides methane, IR1 and DR1 grew on methanol as a sole carbon source at concentrations between 0.2% and 2% v/v. Growth on methanol was limited, however, with cultures reaching a maximum absorbance at 540 nm of 0.5 after three days when grown on methanol, compared with a maximum absorbance at 540 nm of 1.0 when grown on methane. Higher concentrations of methanol (1% v/v and above) gave a lower absorbance at 540 nm of 0.3. This indicates that although IR1 and DR1 can utilize methanol, higher concentrations have an inhibitory effect on the growth of the cells. There was no growth of IR1 and DR1 on any of the methyl compounds tested. IR1 and DR1 also showed no growth on proteose peptone or yeast extract. This indicates that both IR1 and DR1 have very strict nutritional requirements for methane and, in common with most validated reports of other methanotrophs, lack the ability to utilise any other carbon compound except methanol.

### 3.10 Growth of IR1 and DR1 on different nitrogen sources

Initial experiments with alternative media to sea water for IR1

<b>CHARACTERISTICS</b>	<b>IR1</b>	<b>DR1</b>
Morphology	motile rod	motile rod
Size ( $\mu\text{m}$ )	1.4 x 0.8	1.3 x 0.8
Gram stain	-	-
Catalase	+	+
Oxidase	+	+
Catalase	+	+
Rosette formation	-	-
Vitamin requirement	none	none
Specific growth rate (with methane, 30°C 1.5% NaCl, pH 7.6)( $\mu$ )	0.23 hr <sup>-1</sup>	0.20 hr <sup>-1</sup>
Alternative substrate	methanol	methanol
Temperature optimum	30°C	30°C
Growth at 37°C	+	-
pH optimum	7.6	7.6
NaCl optimum	1.5% (w/v)	1.5% (w/v)
DNA composition (%G+C)	49%	50%
Formaldehyde fixation pathway	RuMP	RuMP

**Table 3.5:** Characteristics of marine isolates IR1 and DR1. See text for full details.



CARBON COMPOUND	GROWTH OF IR1	GROWTH OF DR1
Methane sulphonate	no	no
Dimethyl sulphide	no	no
Dimethyl disulphide	no	no
Methane phosphonate	no	no
Carbonyl sulphide	no	no
Trimethylamine	no	no
Dimethylamine	no	no
Monomethylamine	no	no
Proteose peptone	no	no
Nitromethane	no	no
0.2% Methanol	yes	yes
0.5% Methanol	yes	yes
1% Methanol	yes	yes
2% Methanol	slight growth	slight growth
yeast extract	no	no

Table 3.6: Growth of isolates IR1 and DR1 on carbon compounds other than methane. Both isolates show no growth on any other compound except methanol up to a concentration of 2% v/v.

and DR1 showed that ANMS, with the addition of sodium chloride, phosphate and vitamin supplements, was a suitable artificial growth medium for both isolates. This indicated that IR1 and DR1 could utilise either ammonium chloride or potassium nitrate or both as nitrogen sources. This led to further investigation of the utilization of various nitrogen-containing compounds as sole nitrogen sources. The compounds tested and the presence or absence of growth when used as a sole nitrogen source for IR1 and DR1 is shown in table 3.7a. Interestingly, it was observed that neither isolate showed growth on ammonium chloride as sole nitrogen source at a concentration of 0.1% w/v. Ammonium chloride was not toxic to the cells, as growth was clearly seen to be unaffected when the cells were grown on ANMS (containing 0.05% w/v  $\text{NH}_4\text{Cl}$  and 0.05% w/v  $\text{KNO}_3$ ). Whittenbury et al (1970a; b) reported that growth of some methanotrophs was inhibited by concentrations of  $\text{NH}_4\text{Cl}$  higher than 0.05% (w/v) and the experiment was repeated using media containing 0.05% w/v  $\text{NH}_4\text{Cl}$ . Growth was again not observed and it seems that IR1 and DR1 cannot utilize ammonium chloride as a sole nitrogen compound. Growth on both 0.1 % and 0.05% w/v  $\text{KNO}_3$ , however, was vigorous with cultures of both IR1 and DR1 reaching an absorbance at 540 nm of 1.0. This possibly reflects the environment from which IR1 and DR1 were isolated as sea water has relatively high concentrations of nitrate and little free-ammonia (Lees et al, 1991).

The ability of IR1 and DR1 to fix nitrogen ( $\text{N}_2$ ) was also tested. Flasks were set up as described in section 2.4 and incubated. Neither IR1 or DR1 demonstrated the ability to fix nitrogen even under reduced  $\text{O}_2$  tensions.

	IR1	DR1
0.05% $KNO_3$ + 0.05% $NH_4Cl$	+++	+++
0.1% $KNO_3$	+++	+++
0.1% $NH_4Cl$	-	-
0.05% $KNO_3$	+++	+++
0.05% $NH_4Cl$	-	-
NO FIXED N SOURCE	-	-

Table 3.7a: Growth of IR1 and DR1 on different nitrogen sources. No growth was observed when  $NH_4Cl$  was used as the sole nitrogen source but when  $KNO_3$  was present in the medium, observed growth was rapid at concentrations of 0.1% (w/v) and 0.05% (w/v).  $NH_4Cl$  did not appear to inhibit growth of IR1 and DR1 when  $KNO_3$  was also present in the medium.

The growth of the isolates on different amino acids as sole nitrogen sources was also investigated. The presence or absence of growth on the amino acids tested is shown in table 3.7b. The pattern of growth is similar to that observed in Methylococcus capsulatus (Bath) (Murrell, 1981).

### 3.11 Alternative ion donors to sodium chloride

The growth of the isolates IR1 and DR1 was tested on media in which sodium chloride had been replaced with an equal amount of an alternative ion donor. The results obtained are shown in table 3.8.

### 3.12 Optimum conditions for growth of IR1 and DR1

The temperature, pH and sodium chloride concentrations for optimum growth of IR1 and DR1 were determined. Growth experiments were performed as described in section 2.4. Representative growth curves are illustrated in figs. 3.5, 3.6 and 3.7. Doubling times are shown in table 3.9.

The optimum temperature for growth of IR1 was calculated to be 30°C, with a doubling time of 3.0 hr. For DR1, the optimum temperature for growth was also 30°C, with a doubling time of 3.4 hr. At 15°C and 37°C, growth of both IR1 and DR1 was very slow, with a doubling time for IR1 at 15°C of 15 hr and at 37°C a doubling time of 13 hr. DR1 did not grow at 15°C during the course of the experiment, and stopped growing after 36 hours at 37°C.

AMINO ACID	GROWTH OF IR1	GROWTH OF DR1
L-glutamic acid	++	++
L-proline	++	++
L-valine	++	++
L-glutamine	++	++
L-methionine	-	-
L-arginine	+++	+++
L-asparagine	+++	+++
L-cysteine	+	+
L-serine	+++	+
L-glycine	-	-
L-phenylalanine	+++	++
Nitrate media	+++	+++

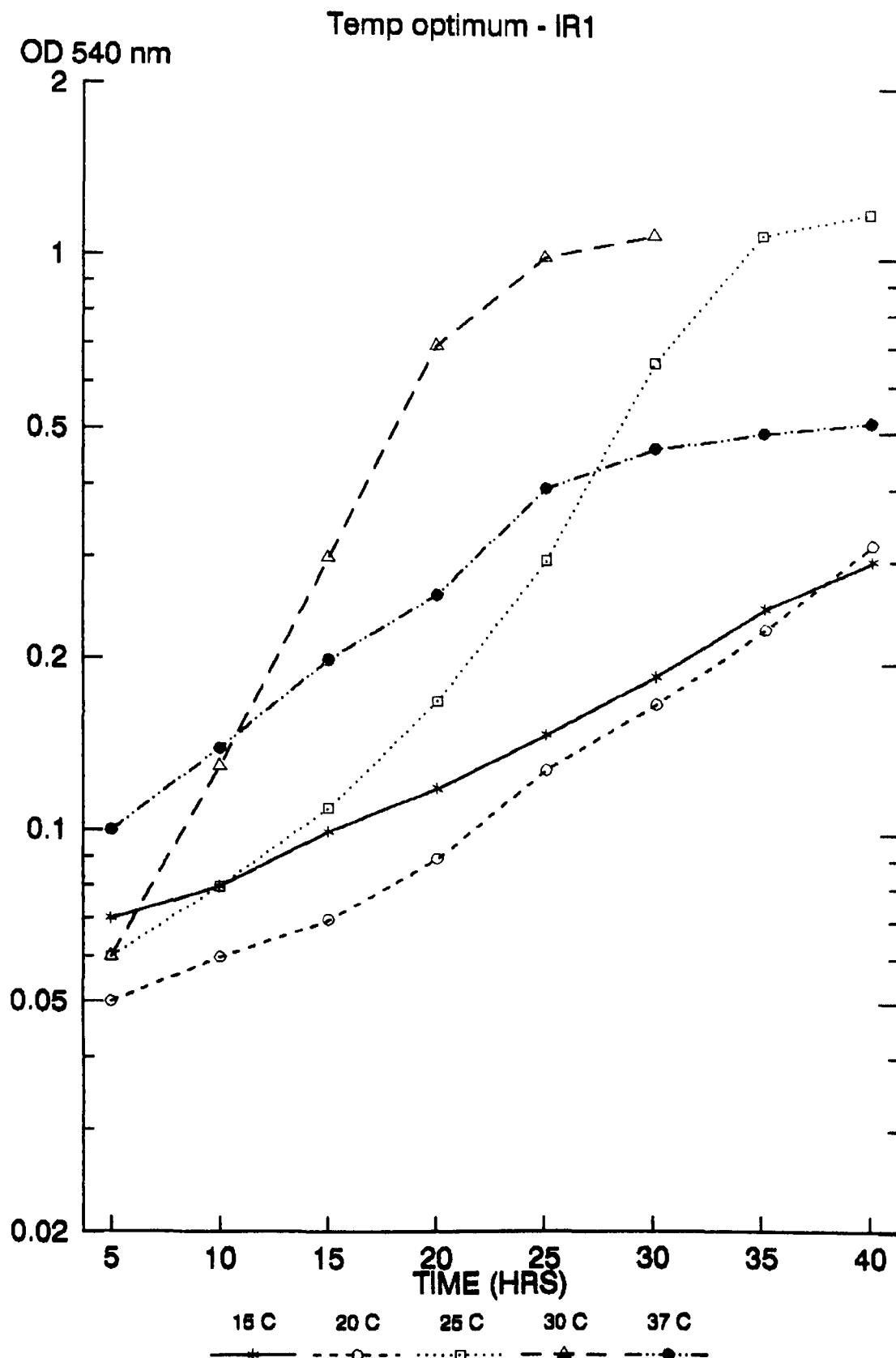
Table 3.7b: The growth of the isolates IR1 and DR1 on a range of amino acids as sole nitrogen sources. The amino acids were added to a final concentration of  $1 \text{ g.l}^{-1}$  to nitrogen-free media. The growth of the cultures was quantified by measuring the absorbance of the culture at 540 nm. This is represented by symbols as is shown below:

- no growth  
+ 0.2 - 0.4  
++ 0.4 - 0.6  
+++ 0.6 - 0.8

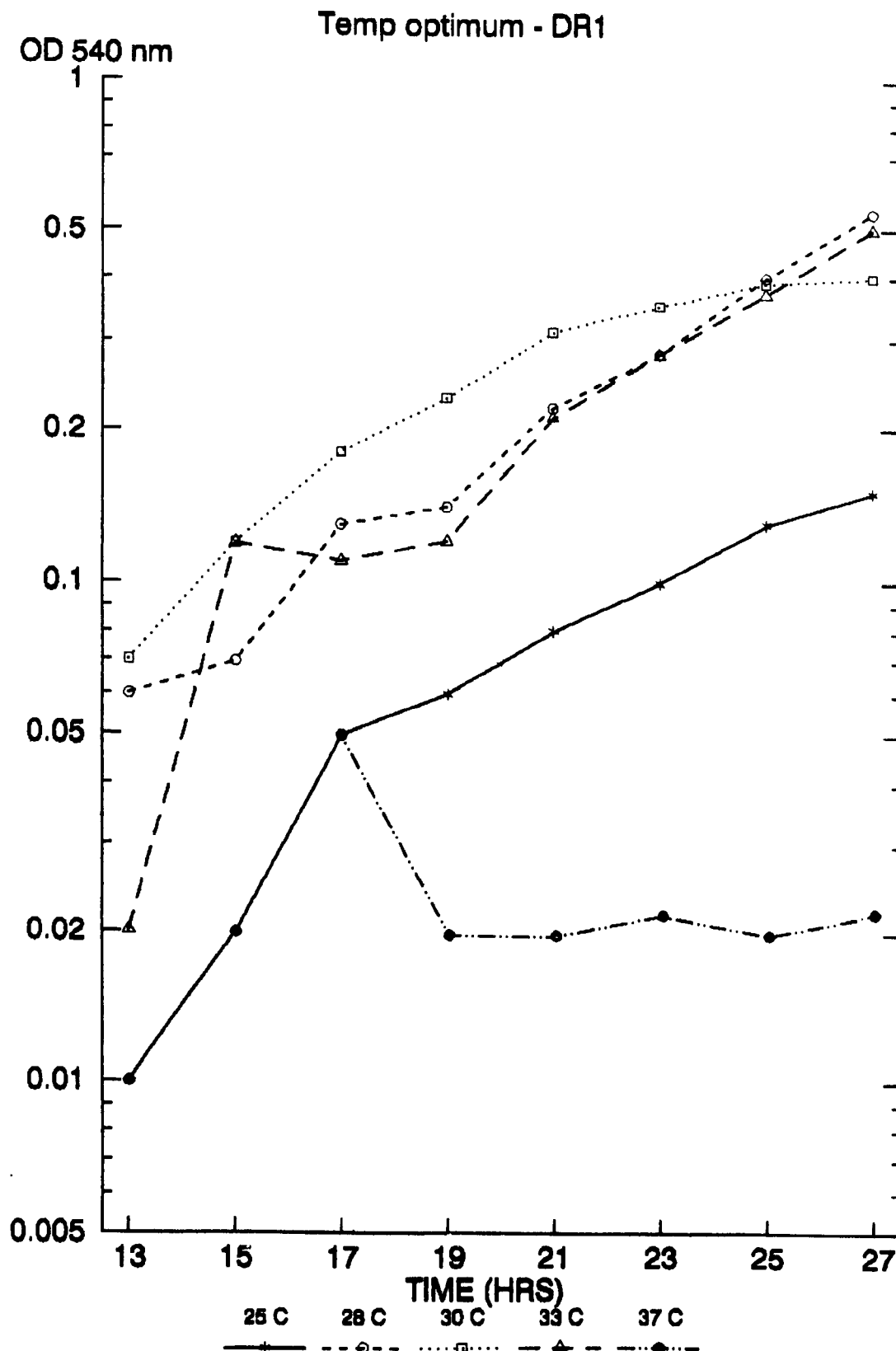
<i>ION DONOR</i>	<i>IR1</i>	<i>DR1</i>
<i>Magnesium chloride</i>	-	-
<i>Sodium fluoride</i>	+	+
<i>Lithium chloride</i>	+	+
<i>Calcium chloride</i>	++	++
<i>Potassium bromide</i>	-	-
<i>Potassium chloride</i>	-	-
<i>Sodium chloride</i>	+++	+++

*Table 3.8: Growth of isolates IR1 and DR1 on ion donors other than sodium chloride. The ion donors were added at a concentration of 1.5% w/v. The growth of the cultures was assessed by the absorbance at 540 nm and is shown as symbols in the table. The absorbance represented by each symbol is shown below:*

-      no growth  
+      0.2 - 0.4  
++     0.4 - 0.6  
+++    0.6 - 0.8

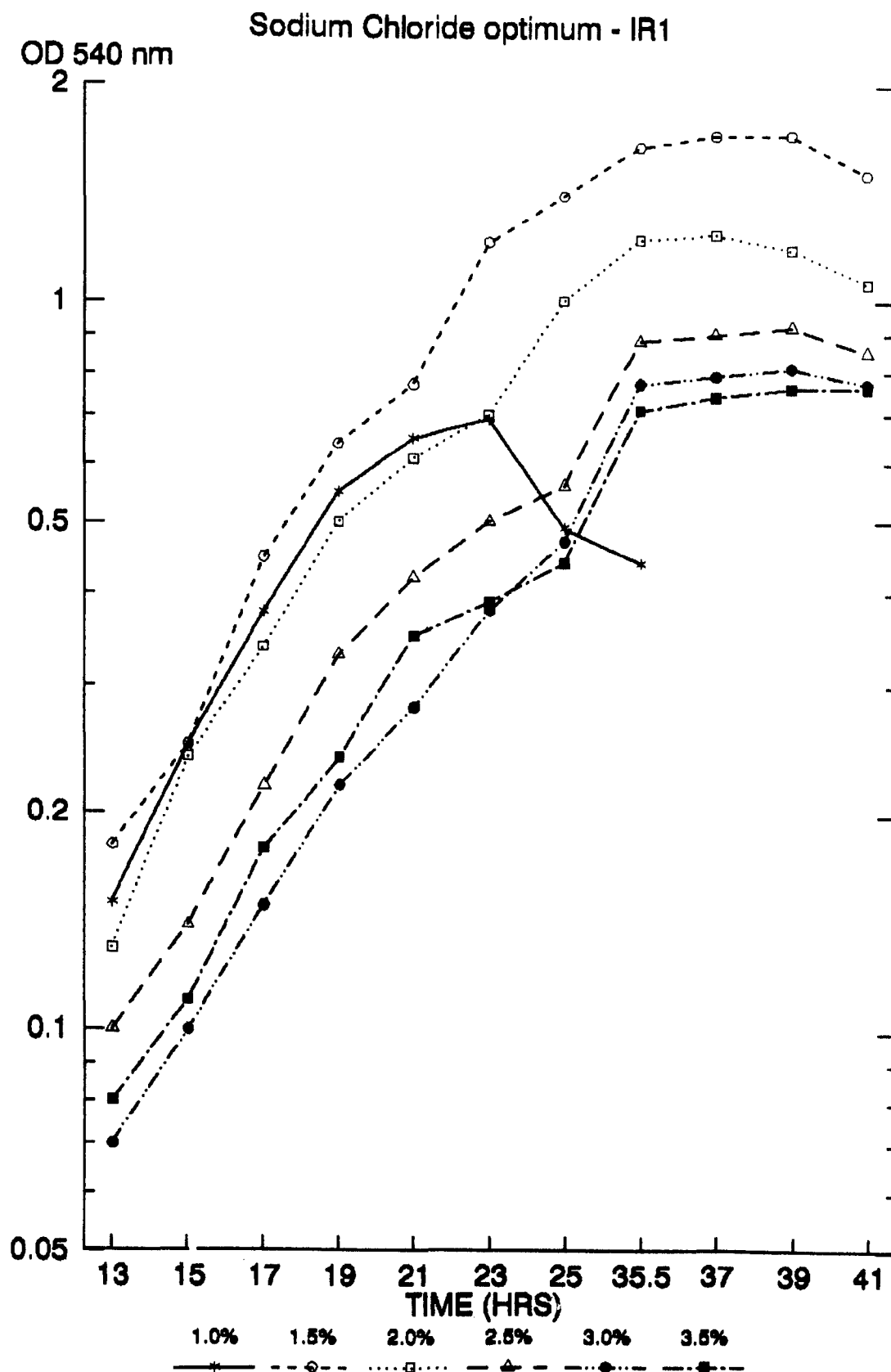


**Fig. 3.5a** : Representative growth curves showing the absorbance at 540 nm of cultures of IR1 grown at different temperatures. For doubling times see table 3.9.

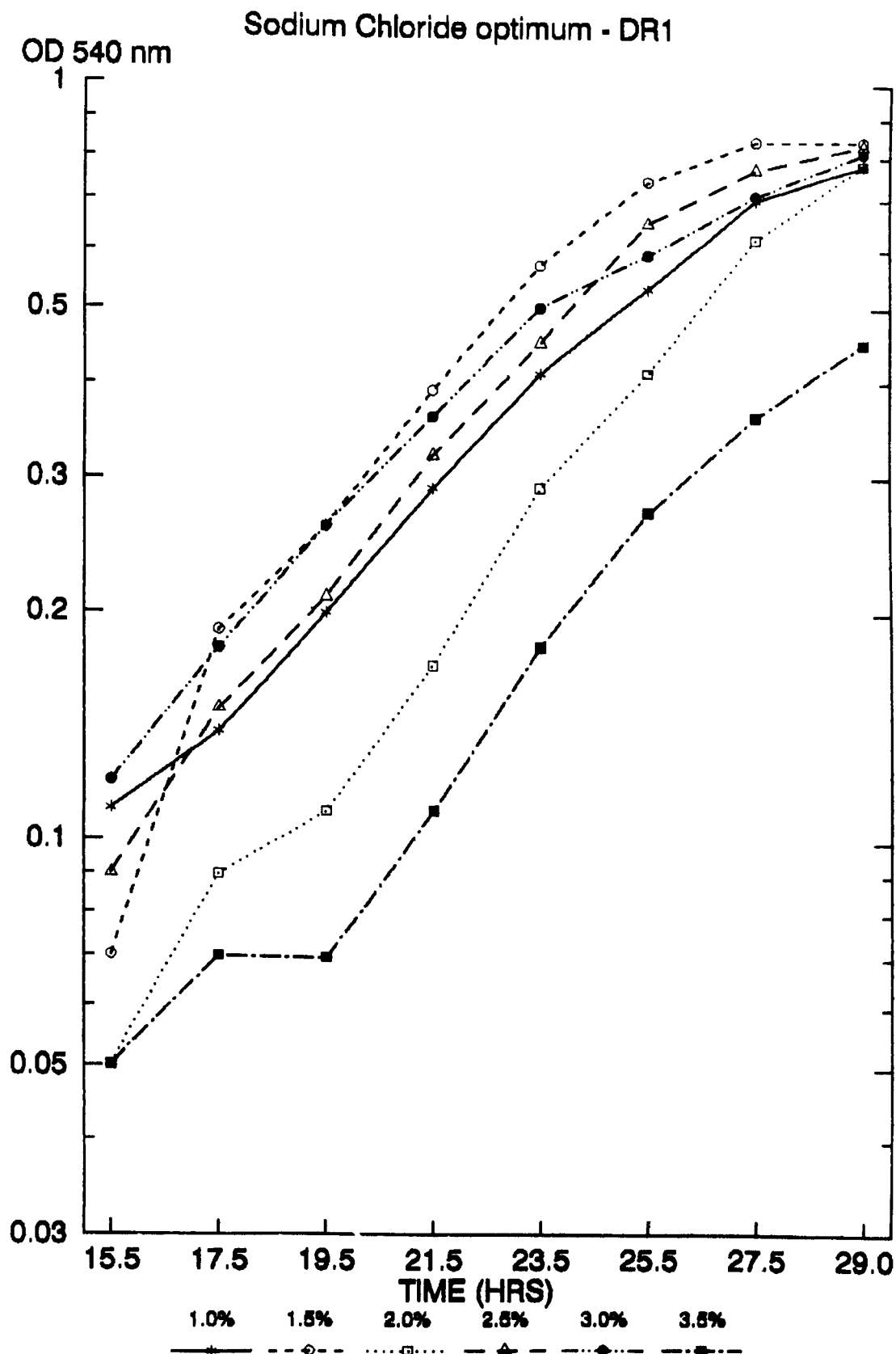


**Fig. 3.5b** : Representative growth curves showing the absorbance at 540 nm of cultures of DR1 grown at different temperatures. For doubling times see table 3.9.

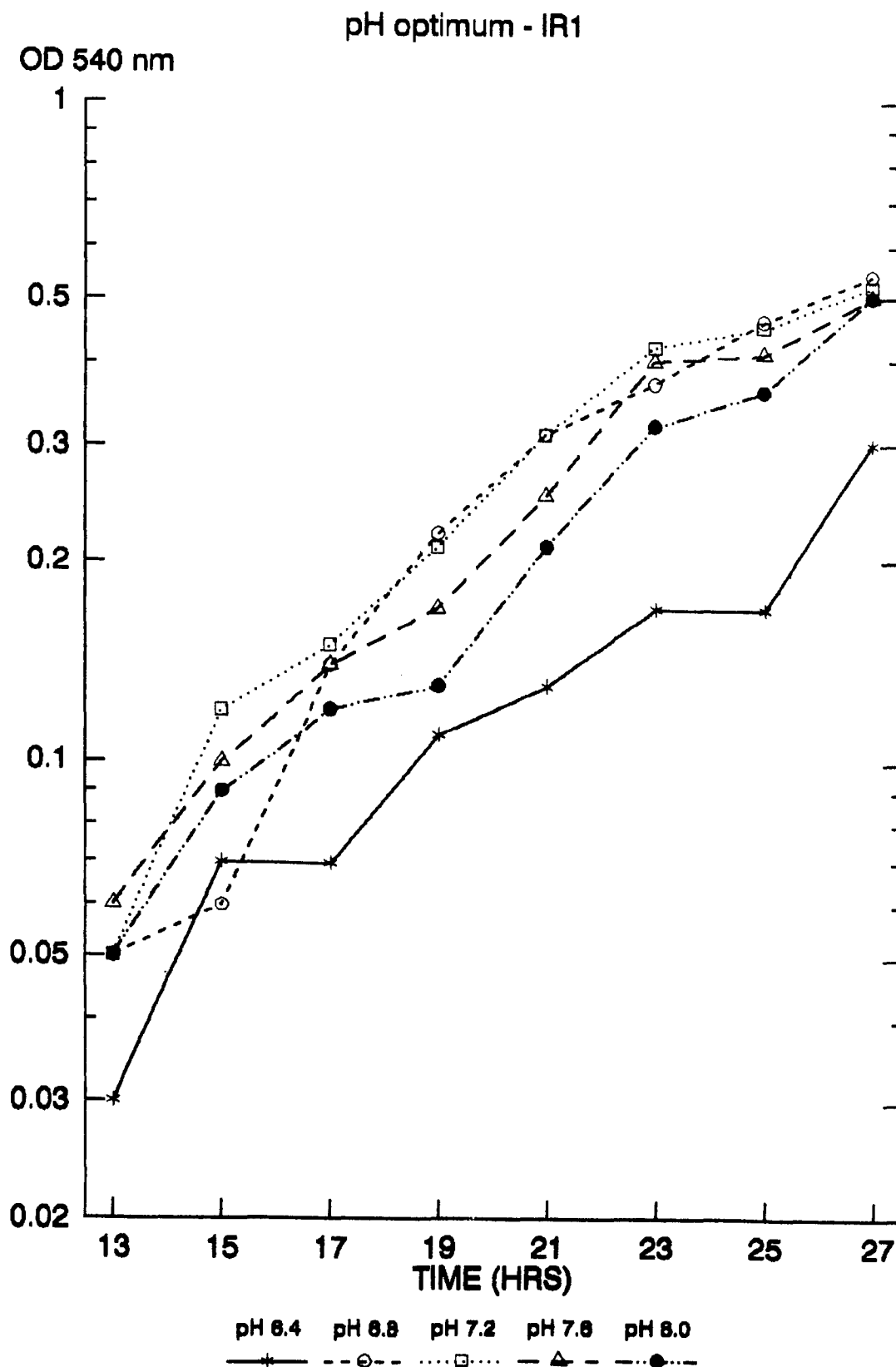




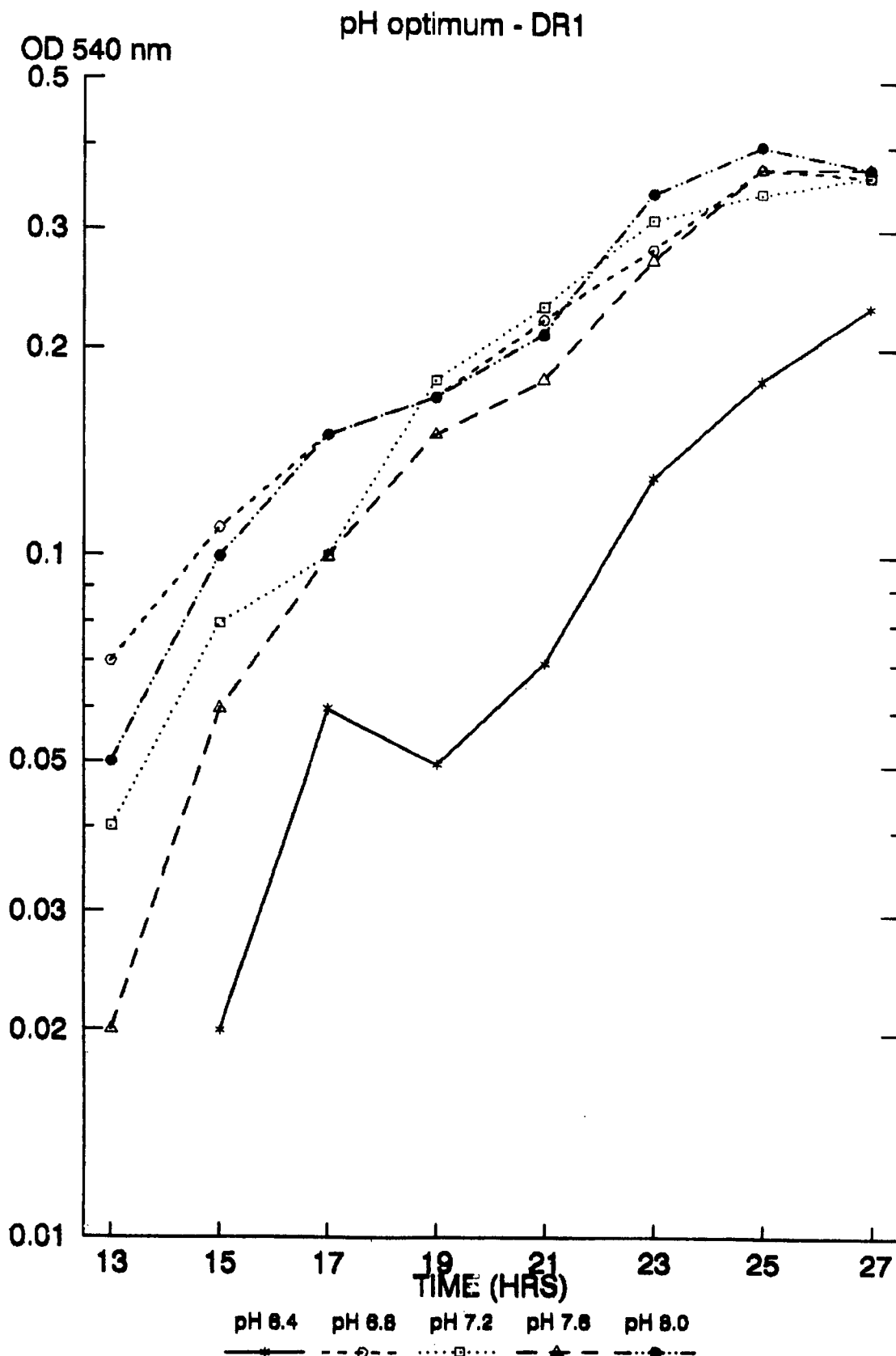
**Fig. 3.6a** : Representative growth curves showing the absorbance at 540 nm of cultures of IR1 grown at different salt concentrations. For doubling times see table 3.9.



**Fig. 3.6b** : Representative growth curves showing the absorbance at 540 nm of cultures of DR1 grown at different salt concentrations. For doubling times see table 3.9.



**Fig. 3.7a** : Representative growth curves showing the absorbance at 540 nm of cultures of IR1 grown at different pH values. For doubling times see table 3.9.



**Fig. 3.7b** : Representative growth curves showing the absorbance at 540 nm of cultures of DR1 grown at different pH values.

For doubling times see table 3.9.

**IR1**

PARAMETER	VALUE	DOUBLING TIME
Temperature (at 1.5% NaCl and pH 7.6)	15°C	15 hr
	20°C	11.5 hr
	25°C	5 hr
	30°C	3.5 hr
	37°C	13 hr
Sodium chloride (at 30°C and pH 7.6)	0.5%	5.0 hr
	1.0%	3.25 hr
	1.5%	3.0 hr
	2.0%	3.25 hr
	2.5%	4.25 hr
	3.0%	4.25 hr
	3.5%	4.25 hr
	4.0%	10 hr
pH value (at 1.5% NaCl and 30°C)	6.4	5.6 hr
	6.8	4.8 hr
	7.2	4.7 hr
	7.6	4.6 hr
	8.0	5.8 hr

**DR1**

PARAMETER	VALUE	DOUBLING TIME
Temperature (at 1.5% NaCl and pH 7.6)	25°C	5.5 hr
	28°C	4.65 hr
	30°C	3.8 hr
	33°C	4.5 hr
	37°C	no growth
Sodium chloride (at 30°C and pH 7.6)	0.5%	7.5 hr
	1.0%	3.5 hr
	1.5%	3.5 hr
	2.0%	3.4 hr
	2.5%	3.4 hr
	3.0%	3.4 hr
	3.5%	3.5 hr
	4.0%	5.9 hr
pH value (at 1.5% NaCl and 30°C)	6.4	5.2 hr
	6.8	5.2 hr
	7.2	4.4 hr
	7.6	3.8 hr
	8.0	5.2 hr

**Table 3.9: Doubling times of IR1 and DR1 when grown in varying conditions. The optimum value for each of three parameters tested (temperature, sodium chloride concentration and pH values) was determined as the value that allowed the quickest doubling time.**

The sodium chloride concentration tolerance of IR1 and DR1 was found to be between 0.5% and 4.5%. As sea water has a sodium chloride concentration of between 3.2 and 3.8% this is likely to indicate that these isolates are typical of true marine strains. The marine methanotrophs described by Lidstrom (1988) demonstrated a salt tolerance of between 0.5% and 1.5%. This is lower than expected for marine bacteria, but as Lidstrom's isolates were obtained from a sewage outfall, it is likely that the sodium chloride concentration at the sample site is lower. IR1 and DR1 have a similar range to that seen in Methylomonas pelagica described by Sieburth et al (1987) which were found to have a sodium chloride tolerance range of 0.4% - 3.2%.

As a control experiment, a selection of freshwater and terrestrial methanotrophs from the Warwick culture collection were inoculated into ANMS media containing 1.5% sodium chloride. The organisms tested are shown in table 3.10. None of these grew in the presence of sodium chloride.

The optimum pH required for growth by both isolates was found to be 7.6, although a pH value of between 6.4 and 8.0 was found to support rapid growth. The pH of the growth medium of 7.6 is higher than that used for fresh water methanotrophs (For example, Methylococcus capsulatus (Bath) has an optimum pH value for growth of 6.8). As the pH of sea water is between 7.5 and 8.5, this higher value is not unexpected

### 3.13 Carbon assimilation pathway enzymes

The enzymes involved in carbon assimilation can help the classi-

<b>METHANOTROPH</b>	<b>ANMS</b>	<b>ANMS + NaCl</b>
A4	***	-
Y	***	-
OB3b	***	-
OBBP	***	-
5	***	-
PM	***	-
A20	***	-

Table 3.10: The growth of culture collection strains of methanotrophs isolated from a range of terrestrial and fresh water environments when subcultured into medium containing sodium chloride. Normal growth is represented by (\*\*\*), no growth is shown by (-). Methanotrophs tested are as follows:

*Methylomonas methanica* A4  
*Methylobacter capsulatus* Y  
*Methylosinus trichosporium* OB3b  
*Methylocystis parvus* OBBP  
*Methylosinus sporium* 5  
*Methylomonas methanica* PM  
*Methylomonas agile* A20

fication of methanotrophic bacteria into Type I or Type II methanotrophs. There are two pathways of carbon assimilation used by methanotrophs: the serine pathway and the ribulose monophosphate pathway. These are described in detail in section 1.5.1. Cell-free extracts from IR1 and DR1 were assayed (as described in materials and methods) to investigate the activity of key enzymes from both pathways. The results are presented below.

Hexulose phosphate synthase is the enzyme which catalyses the first step in the ribulose monophosphate pathway, which is used by Type I methanotrophs to assimilate carbon (in the form of formaldehyde) into the cell (see section 1.5.1). The activities obtained for IR1 and DR1 are shown in table 3.11. Initial activities of hexulose phosphate synthase were very low. However, by careful treatment of the cell-free extract, higher values were obtained. Great care was taken to avoid the extract warming to above 4°C and the extract was used as speedily as possible. The results obtained are still much lower than that obtained with cell-free extract from Methylococcus capsulatus (Bath) which gave a value of 245.07 nmol/min/mg protein. This indicates that the optimal conditions for hexulose phosphate synthase activity in IR1 and DR1 differ from Methylococcus capsulatus (Bath) or that the enzyme in IR1 and DR1 is more unstable.

Hydroxypyruvate reductase is a key enzyme in the serine pathway of carbon assimilation and is described in section 1.5.1. The serine pathway is used by Type II methanotrophs to assimilate carbon from formaldehyde. Cell-free extracts from IR1 and DR1 were assayed on two



ENZYME ASSAYED	IR1	DR1
Alcohol dehydrogenase (PMS-linked)	44.48	87.09
SERINE PATHWAY:		
Hydroxypyruvate reductase	ND	ND
L-serine glyoxylate- amino transferase	ND	ND
Ribulose biphosphate carboxylase	ND	ND
RuMP PATHWAY:		
Hexulose monophosphate synthase	48.66	18
RBP PATHWAY:		
Ribulose biphosphate carboxylase	ND	ND

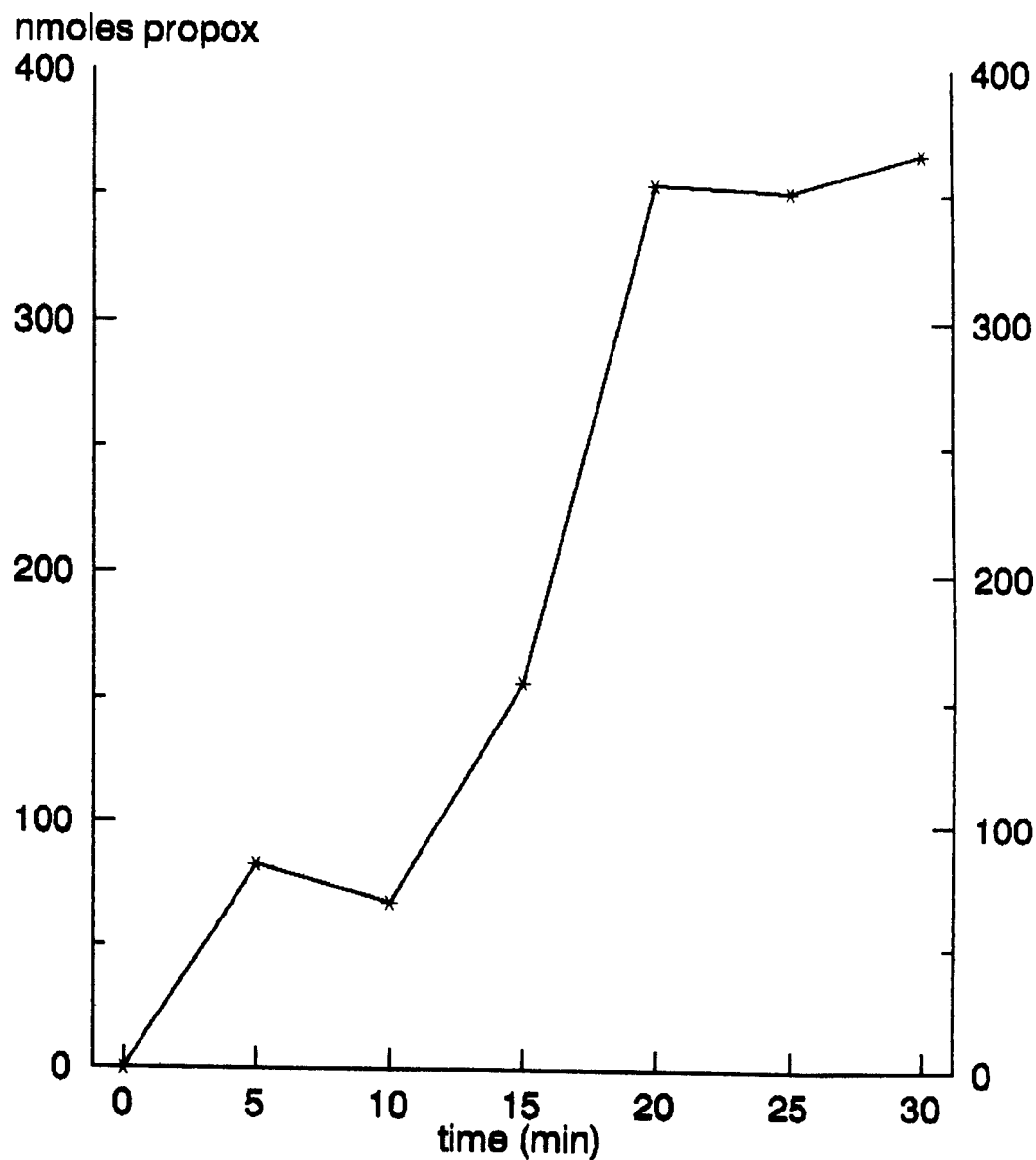
Table 3.11: Carbon assimilation enzyme activities for IR1 and DR1. Rates of activity are expressed in umoles DCPIP reduced/min/mg protein for alcohol dehydrogenase and nmoles/min/mg protein for the other enzymes. Enzymes shown as ND were found to have no detectable activity.

separate occasions using fresh cell-free extract. There was no measurable activity of hydroxypyruvate reductase in either IR1 or DR1 and it is concluded that neither IR1 or DR1 assimilate carbon into the cell by the serine pathway. It is apparent, however, that both IR1 and DR1 assimilate carbon by the ribulose monophosphate pathway.

All Gram negative methanotrophs so far examined convert methanol to formaldehyde by a PMS-linked alcohol dehydrogenase (see section 1.6.2). The activity of PMS-linked alcohol dehydrogenase was investigated in cell-free extracts of IR1 and DR1 as described in section 2.4. The results are shown in table 3.11. Both IR1 and DR1 were found to have low activities for alcohol dehydrogenase when compared with other methanotrophs. IR1 was found to have an activity for alcohol dehydrogenase of 44.48  $\mu\text{mol DCPIP reduced/min/mg protein}$ , whereas DR1 was found to have a rate twice that of IR1 of 87.09  $\mu\text{mol DCPIP reduced/min/mg protein}$ .

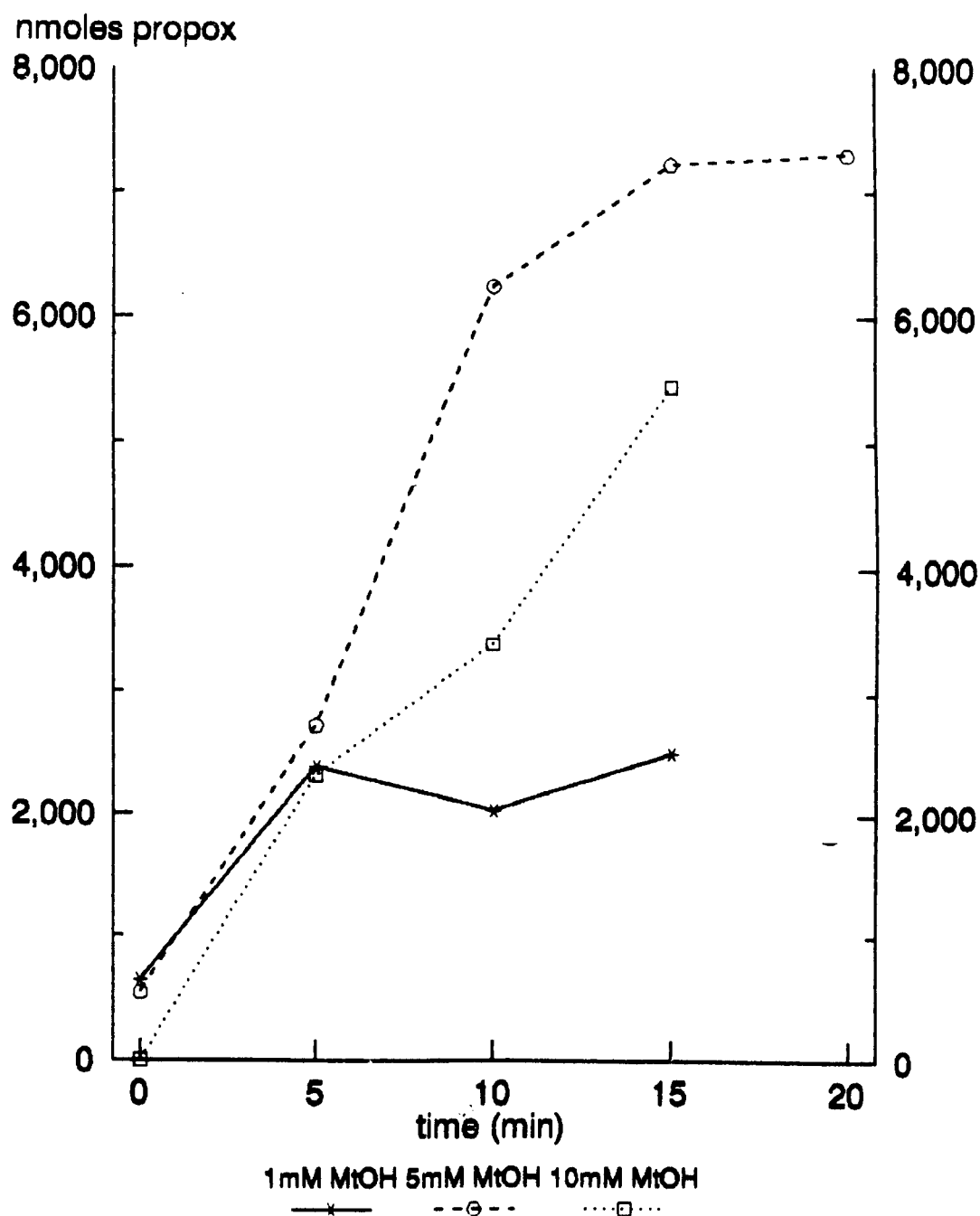
Methane monooxygenase activity in both isolates was investigated by assaying the conversion of propylene to propylene oxide. The assay was performed on whole cells of IR1 and DR1 with and without the addition of methanol and formate, both of which enhance the activity of methane monooxygenase. MMO activities are shown in figs. 3.8a, b & c and 3.9a,b, & c and listed in table 3.12. Both bacteria showed an increase in the activity of methane monooxygenase in the presence of methanol and formate.

Oxidation of propylene to propylene oxide - IR1  
(no additions)



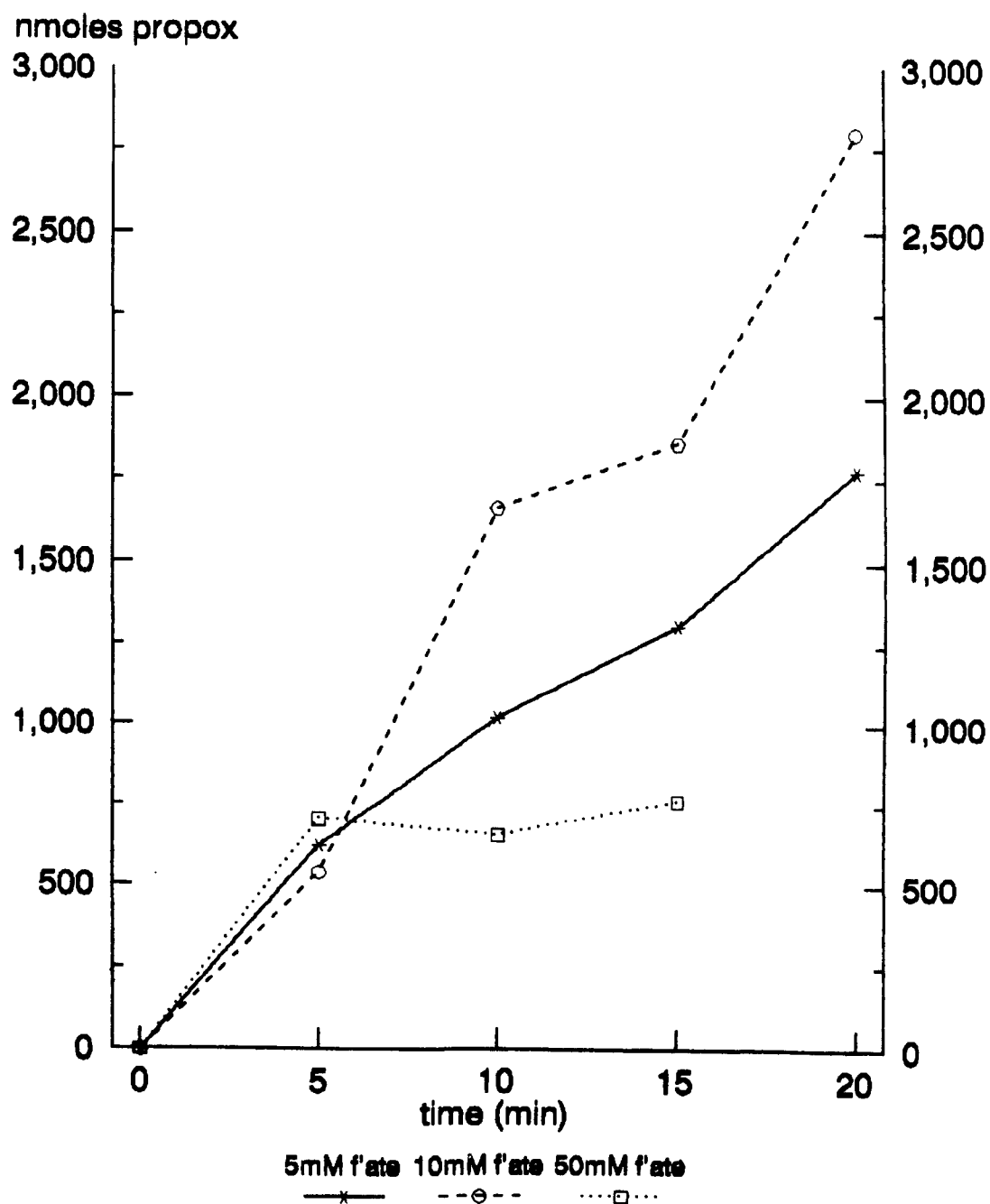
**Fig. 3.8a** : Oxidation of propylene to propylene oxide by IR1 in the presence of no exogenous electron donors. The rate of activity is shown in table 3.12.

Oxidation of propylene to propylene oxide - IR1  
(methanol)



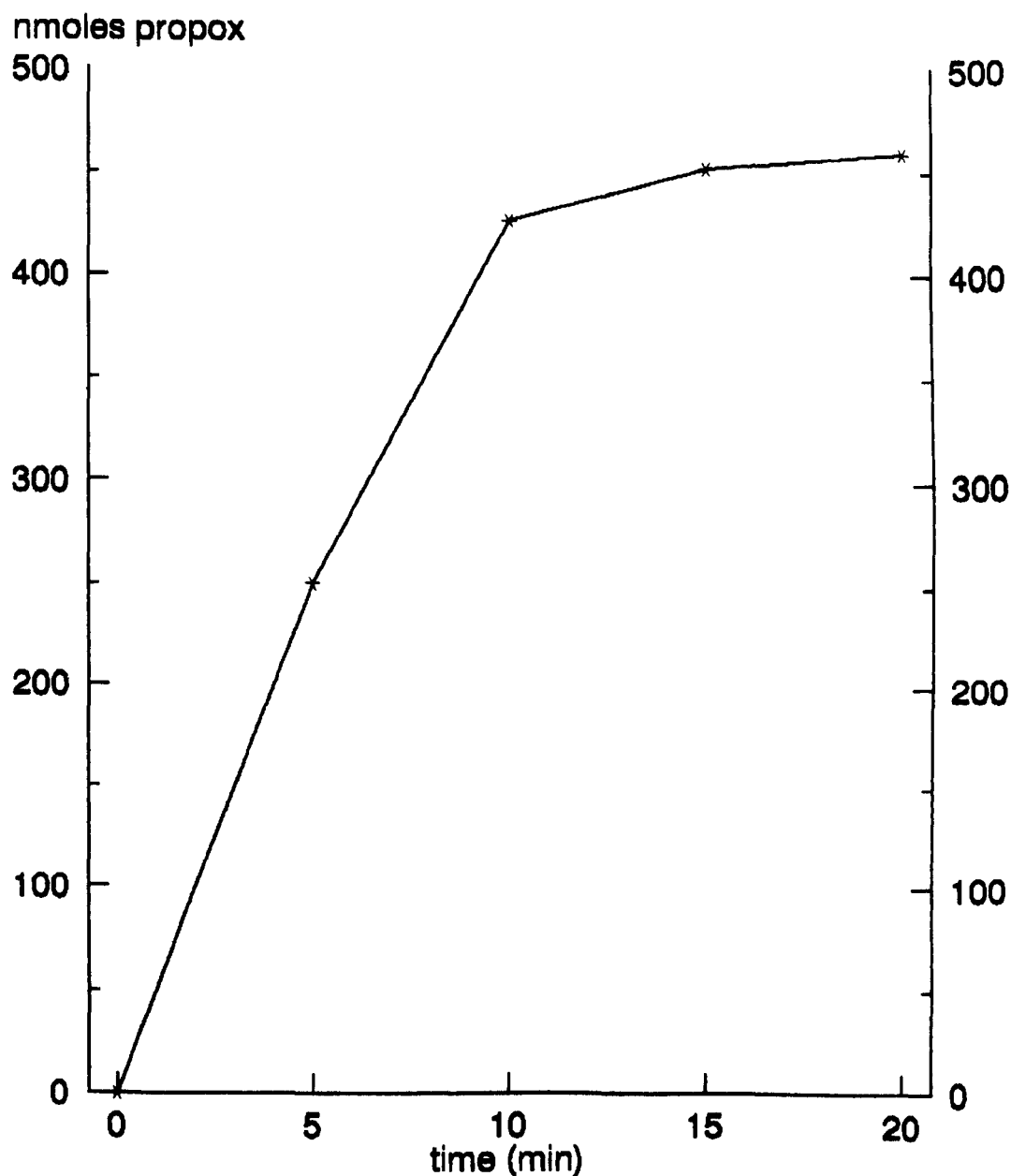
**Fig. 3.8b:** Oxidation of propylene to propylene oxide by IR1 in the presence of methanol as an electron donor. The rate of activity is shown in table 3.12.

**Oxidation of propylene to propylene oxide - IR1  
(formate)**



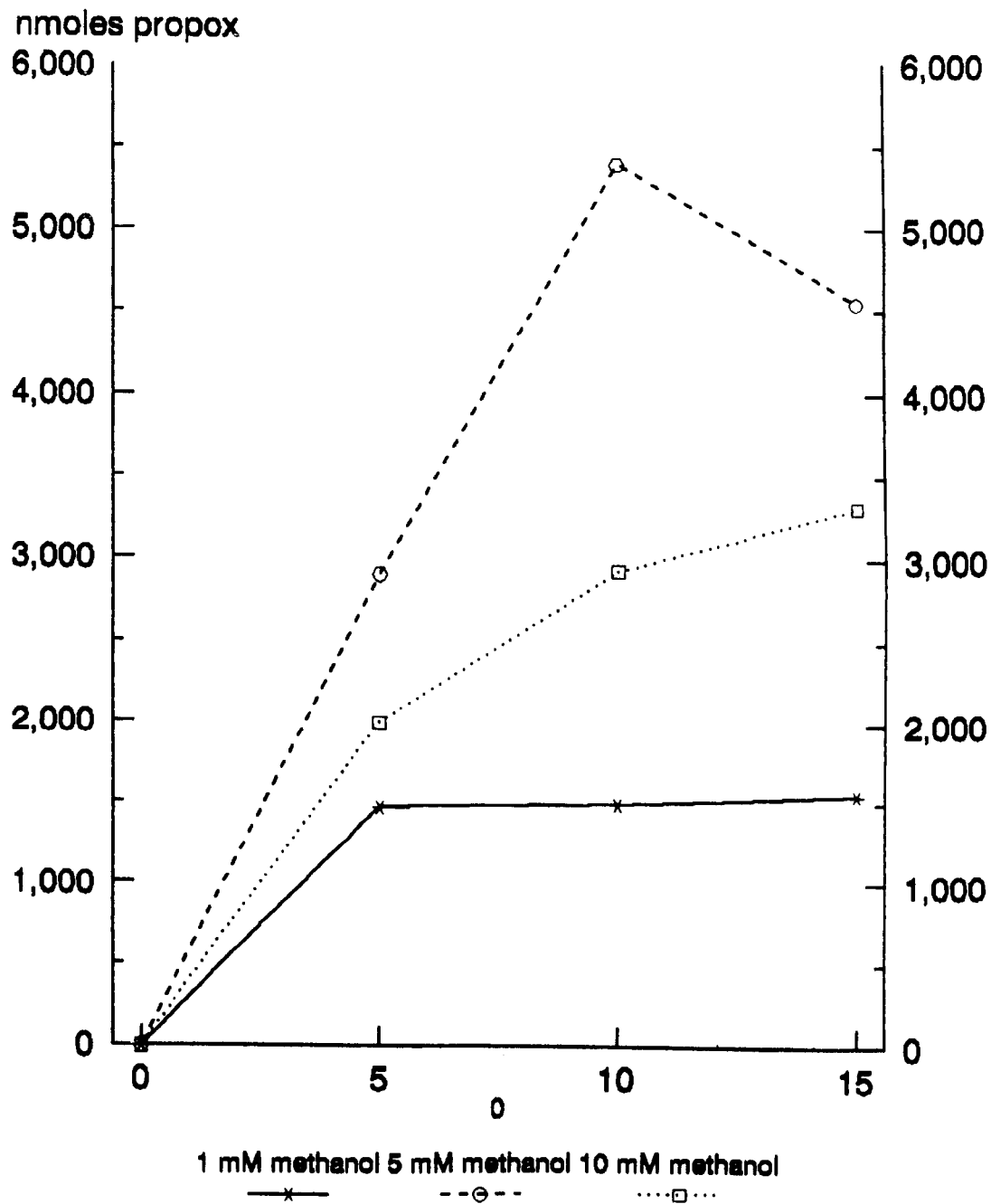
**Fig. 3.8c:** Oxidation of propylene to propylene oxide by IR1 in the presence of formate as an electron donor. The rate of activity is shown in table 3.12.

**Oxidation of propylene to propylene oxide - DR1  
(no addition)**



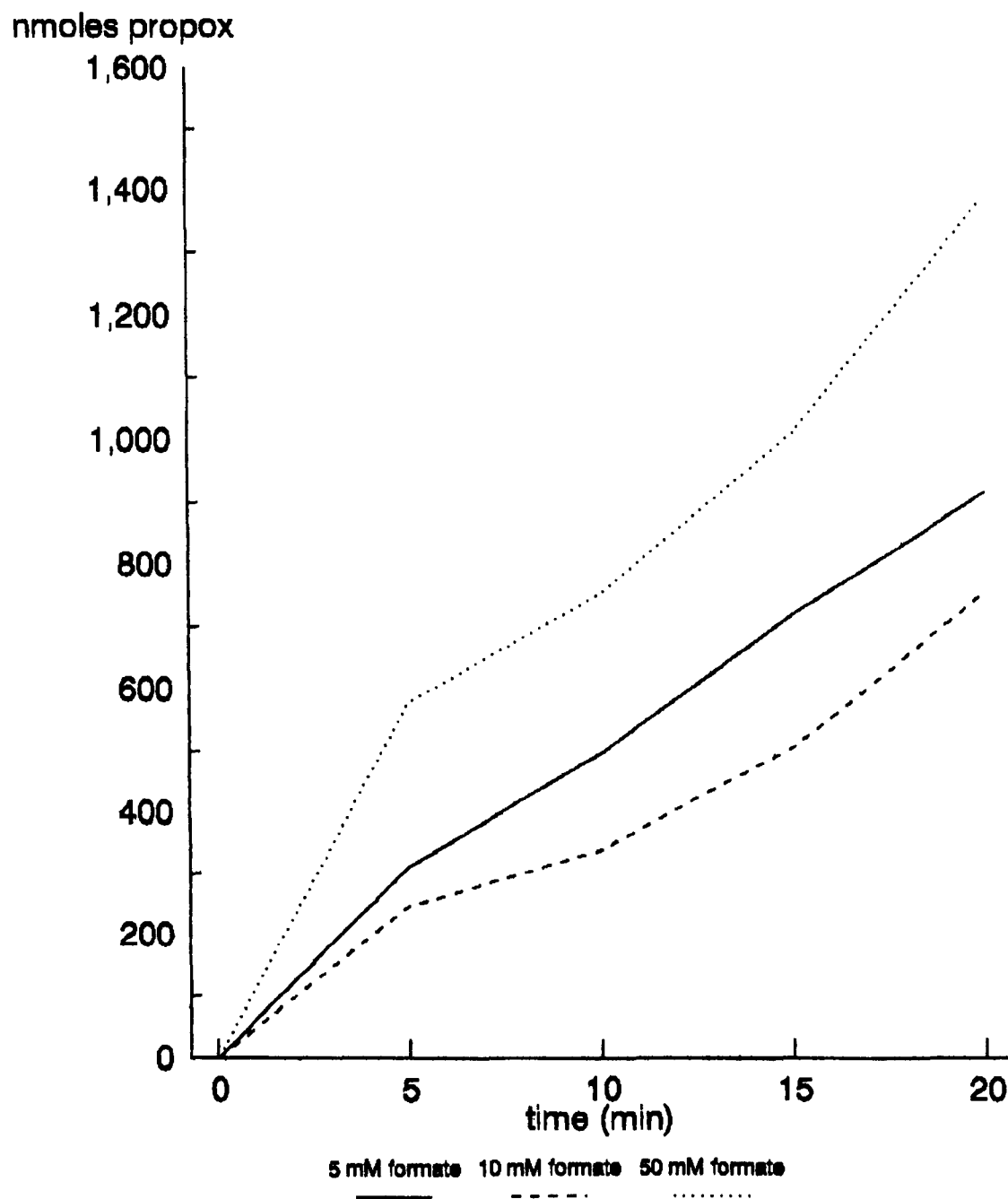
***Fig. 3.9a*** : Oxidation of propylene to propylene oxide by DR1 in the presence of no exogenous electron donors. The rate of activity is shown in table 3.12.

**Oxidation of propylene to propylene oxide - DR1  
(methanol)**



**Fig. 3.9b:** Oxidation of propylene to propylene oxide by DR1 in the presence of methanol as an electron donor. The rate of activity is shown in table 3.12.

Oxidation of propylene to propylene oxide - DR1  
(formate)



**Fig. 3.9c.** Oxidation of propylene to propylene oxide by DR1 in the presence of formate as an electron donor. The rate of activity is shown in table 3.12.



Isolate IR1

<b>ADDITION</b>	<b>OXIDATION RATE (nmoles/min/mg)</b>
none	14.9
1 mM methanol	177
5 mM methanol	480
10 mM methanol	290
5 mM formate	80
10 mM formate	111.4
50 mM formate	80

Isolate DR1

<b>ADDITION</b>	<b>OXIDATION RATE (nmoles/min/mg)</b>
none	44
1 mM methanol	211
5 mM methanol	565
10 mM methanol	344
5 mM formate	40
10 mM formate	73
50 mM formate	40

**Table 3.12: The oxidation of propylene to propylene oxide by the isolates IR1 and DR1. The oxidation was followed over 25 minutes and the rate is expressed in nmoles of propylene oxide formed in 1 minute by 1 mg protein.**

The presence of soluble methane monooxygenase (sMMO) in IR1 and DR1 was investigated by DNA hybridization using a gene probe specific for sMMO from Methylococcus capsulatus (Bath) containing all five structural genes for sMMO. Neither IR1 or DR1 exhibited homology to the sMMO probe even at low stringencies of hybridization allowing approximately 50% base pair mismatch. It is therefore concluded that sMMO is not present in either isolate.

This conclusion was supported by the observation that neither isolate grew in media containing low levels of copper. Low copper is known to be necessary for the switch from particulate to soluble MMO activity (J.C. Murrell, pers. comm.).

Soluble protein extracts from IR1 were separated by polyacrylamide gel electrophoresis and compared to the polypeptide profiles obtained for Methylococcus capsulatus (Bath) (which contains sMMO) and Methylosinus sporium (which contains only pMMO) (fig 3.10). None of the sMMO-specific bands were present in the IR1 extract.

Carbon dioxide assimilation by ribulose 1,5 biphosphate carboxylase (RuBisCo) has been demonstrated in Methylococcus capsulatus (Bath) (Stanley & Dalton, 1982), but it has not been found in any other methanotroph investigated. RuBisCo activity was investigated in IR1 and DR1, but no activity was observed (table 3.11).

### 3.14 Nitrogen assimilation enzymes

As IR1 and DR1 showed no growth on ammonium chloride, it was felt that the nitrogen assimilation enzymes should be investigated. The pathways involved in nitrogen assimilation in bacteria and in parti

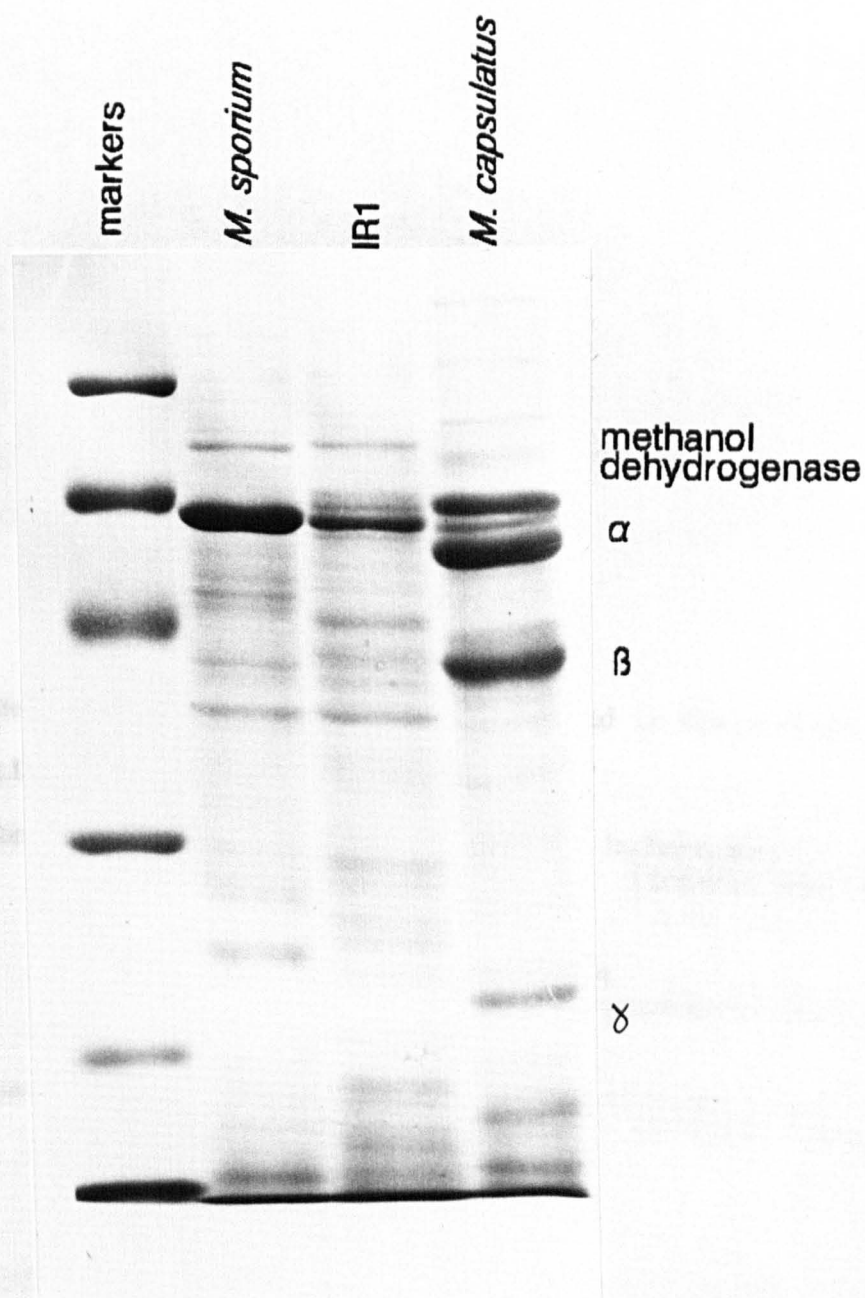
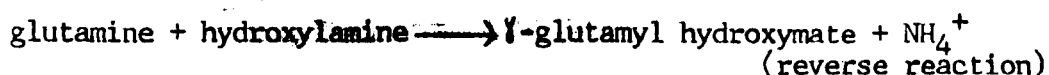
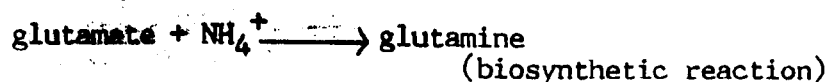
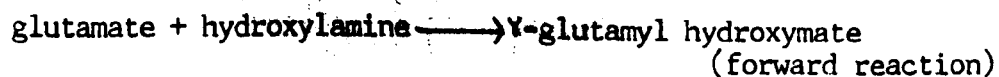


Fig. 3.10 : Polyacrylamide gel showing the major polypeptide bands of *Methylococcus capsulatus* (Bath) *Methylosinus sporium* and isolate IR1. Major sMMO protein bands in are shown. IR1 has a protein profile similar to that of and has none of the sMMO bands present.

cular in methanotrophs are described in detail in section 1.5.2. Four enzymes that were likely to be involved in nitrogen assimilation were examined: glutamine synthetase and glutamate synthase (GS/GOGAT); alanine dehydrogenase and glutamate dehydrogenase. Cell-free extracts from both isolates grown on both ANMS and NMS were assayed for all four enzymes. The rate of activity of each enzyme was calculated and the Michaelis-Menten coefficient ( $K_m$ ) was calculated for glutamine synthetase and glutamate dehydrogenase.

Glutamine synthetase (GS) activity was assayed in three ways: forward reaction, biosynthetic and reverse.



When the isolates were assayed by the forward reaction and the biosynthetic reaction, no GS activity could be measured. The conversion of hydroxylamine and glutamine to  $\gamma$ -glutamyl hydroxamate, however, could be used to obtain GS activity (Murrell & Dalton, 1983) and the results are shown in tables 3.13 and 3.14. The  $K_m$  values for hydroxylamine were also calculated for IR1 and DR1 using the  $\gamma$ -glutamyl transferase assay and are shown in fig. 3.11. These were

found to be high for both isolates when compared with values obtained from other methanotrophs.

The activity of glutamate synthase was assayed in cell-free extracts of IR1 and DR1, grown on both ANMS and NMS. The assay was performed using either NADH or NADPH, tables 3.13 and 3.14. The GOGAT enzyme from both organisms had a higher activity when NADPH was the exogenous reductant and there was no discernible difference between the activities of cells grown on ANMS and those grown on NMS.

Cell-free extracts from ANMS and NMS grown cells of both IR1 and DR1 were assayed for glutamate dehydrogenase activity (tables 3.13 and 3.14). The enzyme had higher activities in assays which were NADPH linked. There was no obvious difference between the activities of ANMS and NMS grown cells. The  $K_m$  values for ammonia of DR1 GDH and IR1 GDH were calculated and found to be low (fig. 3.12)

Alanine dehydrogenase activity was investigated in cell-free extracts from IR1 and DR1 grown on ANMS and NMS (tables 3.13 and 3.14). Levels of activities are all low, with NADH linked activities giving higher rates.

Nitrate reductase activity (which catalyses the conversion of nitrate to ammonia within the cell) was assayed in both IR1 and DR1. The rates of activity for nitrate reductase are shown in table 3.13. No activity was observed for heat-killed cells.

ENZYME	GROWTH MEDIUM	ACTIVITY (IR1)	ACTIVITY (DR1)
GS (transferase assay)	NMS ANMS	157 200	360 200
GOGAT (NADPH-dependent)	NMS ANMS	23 24	23 37
GDH (NADPH-dependent)	NMS ANMS	30 42	61 58
ADH (NADH-dependent)	NMS ANMS	5 11	7 14
Nitrate reductase	ANMS	103	86
Nitrogenase	MS	ND	ND

Table 3.13: Activity of nitrogen assimilation enzymes in isolates IR1 and DR1. The rates shown are the average of two values, each value was calculated from the rate obtained from different initiators (see text). The activities are expressed in nmoles/min/mg protein. The enzymes assayed were as follows:

ADH - Alanine dehydrogenase  
GDH - Glutamate dehydrogenase  
GOGAT - Glutamate synthase  
GS - Glutamine synthetase

ND - Not detected

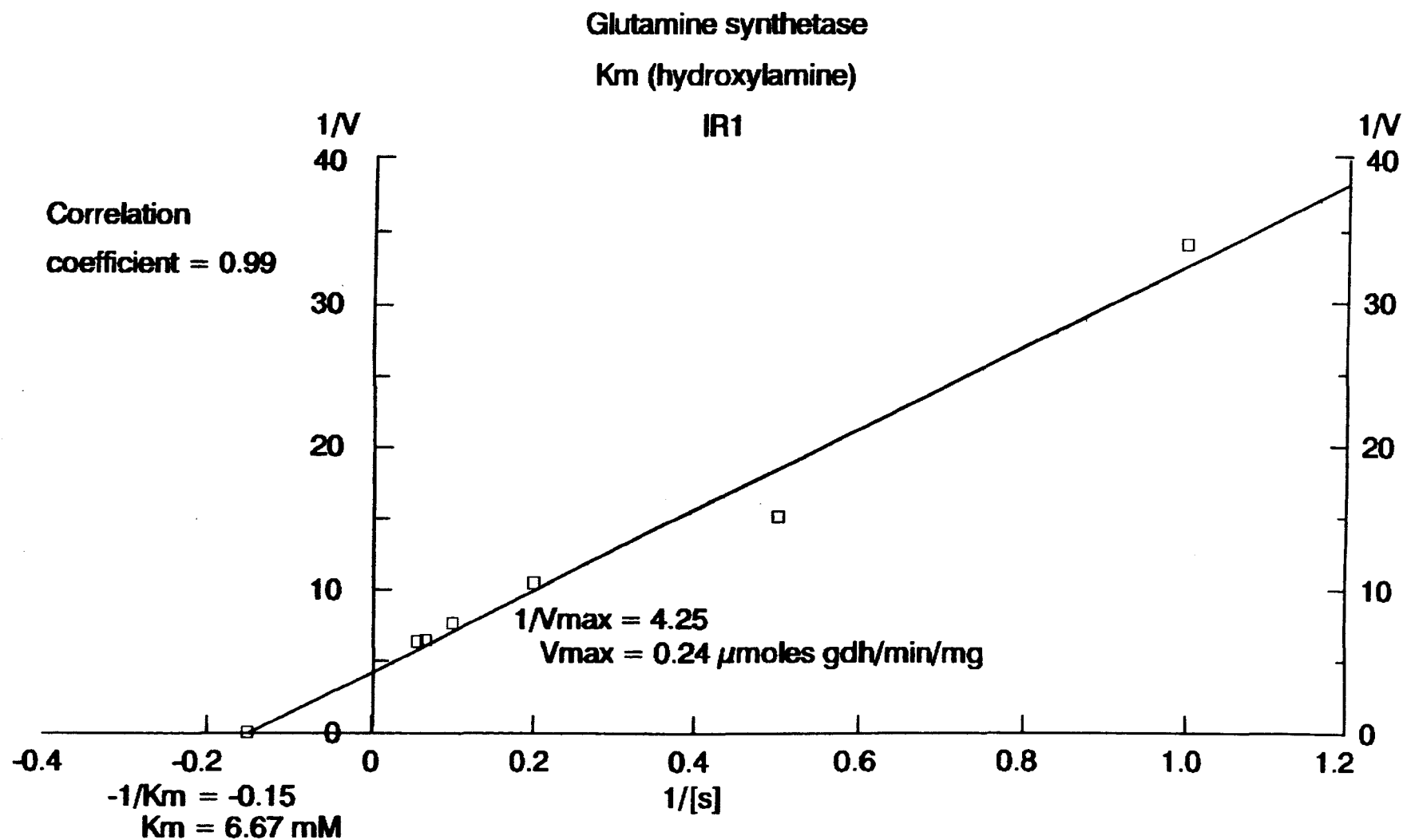
ENZYME	MEDIA	COFACTOR	INITIATOR	ACTIVITY
ADH	NMS	NADH	NH <sub>4</sub> Cl	4.67
		NADH	pyruvate	5.05
		NADPH	NH <sub>4</sub> Cl	0
		NADPH	pyruvate	5.08
ADH	ANMS	NADH	NH <sub>4</sub> Cl	16.0
		NADH	NH <sub>4</sub> Cl	10.0
		NADH	pyruvate	7.70
		NADH	pyruvate	2.9
		NADH	pyruvate	8.33
		NADPH	NH <sub>4</sub> Cl	0
		NADPH	NH <sub>4</sub> Cl	0
		NADPH	pyruvate	0
		NADPH	pyruvate	0
GDH	NMS	NADH	NH <sub>4</sub> Cl	8.74
		NADH	δ-KG	0
		NADPH	NH <sub>4</sub> Cl	29.29
		NADPH	γ-KG	30.14
GDH	ANMS	NADH	NH <sub>4</sub> Cl	18.1
		NADH	NH <sub>4</sub> Cl	5.3
		NADH	δ-KG	0
		NADH	δ-KG	0
		NADPH	NH <sub>4</sub> Cl	26.1
		NADPH	NH <sub>4</sub> Cl	18.12
		NADPH	δ-KG	29.13
		NADPH	γ-KG	57.42
GOGAT	NMS	NADH	glutamate	8.64
		NADH	δ-KG	0
		NADPH	glutamate	20.1
		NADPH	γ-KG	26.05
GOGAT	ANMS	NADH	glutamate	7.53
		NADH	glutamate	2.1
		NADH	δ-KG	2.7
		NADH	γ-KG	0
		NADPH	glutamate	18.23
		NADPH	glutamate	20.8
		NADPH	δ-KG	16.9
		NADPH	γ-KG	30.93

Table 3.14a: Assays of the nitrogen assimilation enzymes in the isolate IR1. Results for the enzymes alanine dehydrogenase (ADH); glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) are shown above. Activities are expressed in nmoles/min/mg protein. Cell-free extract was prepared from cells grown in the presence of nitrate (NMS) or in the presence of ammonia and nitrate (ANMS). The results presented in this table are summarised in table 3.13.

ENZYME	MEDIA	COFACTOR	INITIATOR	ACTIVITY
ADH	NMS	NADH	NH <sub>4</sub> Cl	5.95
		NADH	pyruvate	8.5
		NADPH	NH <sub>4</sub> Cl	0
ADH	ANMS	NADH	NH <sub>4</sub> Cl	15.22
		NADH	NH <sub>4</sub> Cl	7.8
		NADH	pyruvate	7.5
		NADH	pyruvate	12.2
		NADPH	NH <sub>4</sub> Cl	2.12
		NADPH	NH <sub>4</sub> Cl	1.58
		NADPH	pyruvate	0
		NADPH	pyruvate	2.8
GDH	NMS	NADH	NH <sub>4</sub> Cl	6.5
		NADPH	NH <sub>4</sub> Cl	68.0
		NADPH	γ-KG	53.0
GDH	ANMS	NADH	NH <sub>4</sub> Cl	18.73
		NADH	NH <sub>4</sub> Cl	11.2
		NADH	δ-KG	0.9
		NADH	δ-KG	2.65
		NADPH	NH <sub>4</sub> Cl	52.68
		NADPH	NH <sub>4</sub> Cl	20.68
		NADPH	γ-KG	25.7
		NADPH	δ-KG	62.9
GOGAT	NMS	NADH	glutamate	9.0
		NADH	δ-KG	2.9
		NADPH	glutamate	24.01
		NADPH	δ-KG	21.8
GOGAT	ANMS	NADH	glutamate	10.16
		NADH	glutamate	4.1
		NADH	δ-KG	2.64
		NADH	γ-KG	5.96
		NADPH	glutamate	35.94
		NADPH	glutamate	17.86
		NADPH	δ-KG	22.3
		NADPH	δ-KG	38.28

Table 3.14b: Assays of the nitrogen assimilation enzymes in the isolate DR1. Results for the enzymes alanine dehydrogenase (ADH); glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) are shown above. Activities are expressed in nmoles/min/mg protein. Cell-free extract was prepared from cells grown in the presence of nitrate (NMS) or in the presence of ammonia and nitrate (ANMS). The results presented in this table are summarised in table 3.13.





**Fig. 3.11a** Determination of the K<sub>m</sub> for hydroxylamine of the glutamine synthetase from IR1

# Glutamine synthetase

$K_m$  (hydroxylamine)

DR1

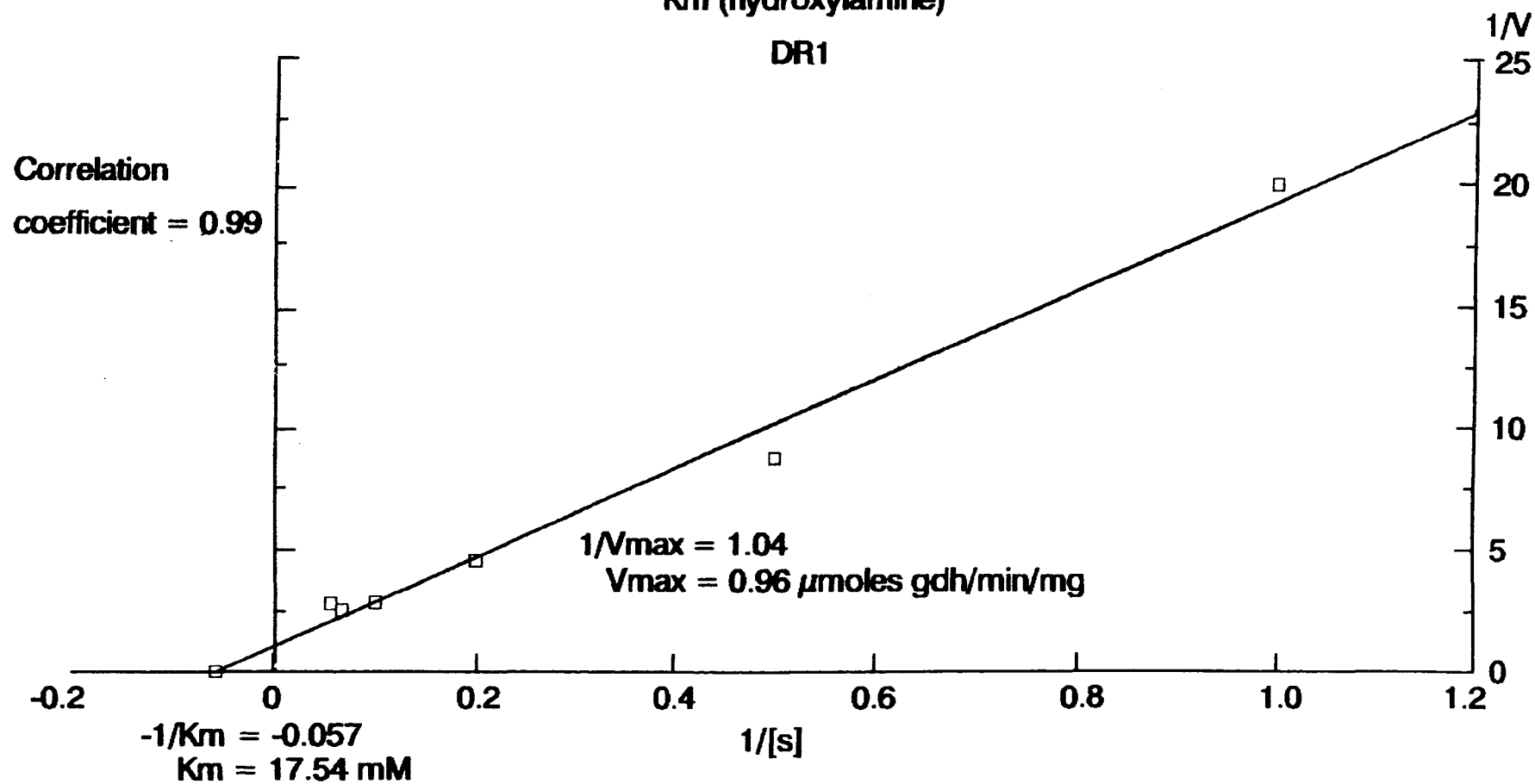
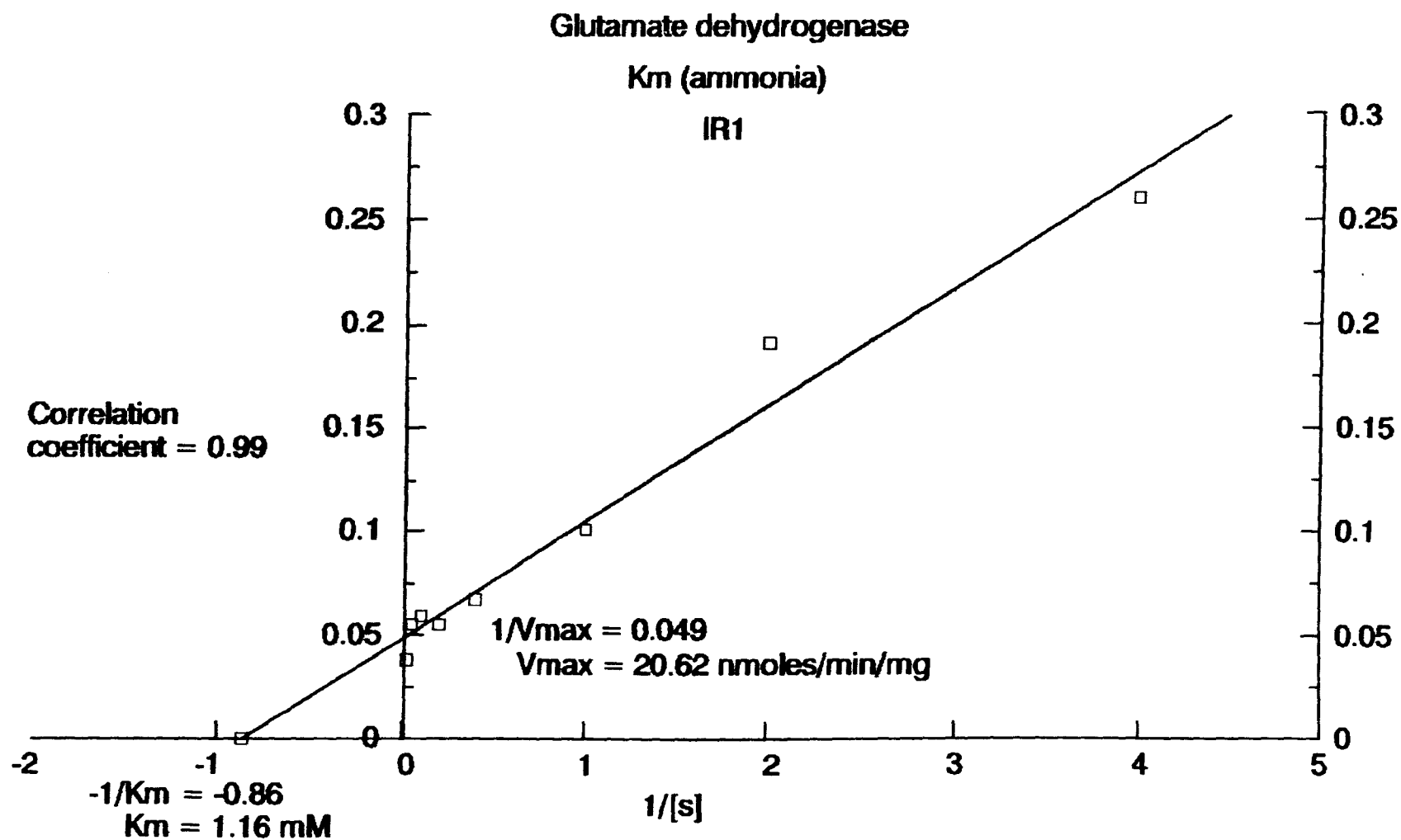
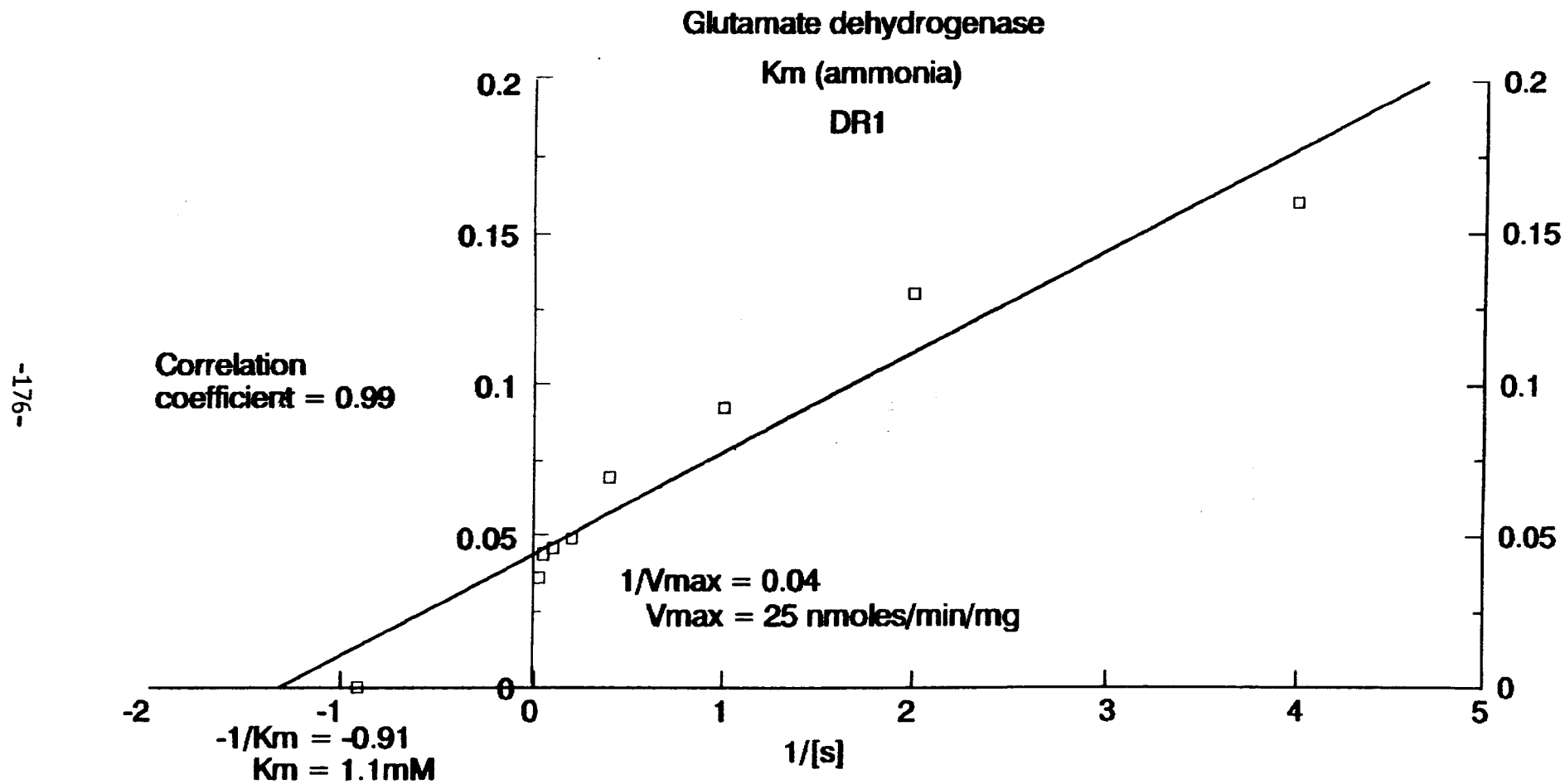


Fig. 3.11b Determination of the  $K_m$  for hydroxylamine of the glutamine synthetase from DR1



**Fig. 3.12a:** Determination of the  $K_m$  for ammonia of the glutamate dehydrogenase from IR1



**Fig. 3.12b: Determination of the K<sub>m</sub> for ammonia of the glutamate dehydrogenase from DR1**

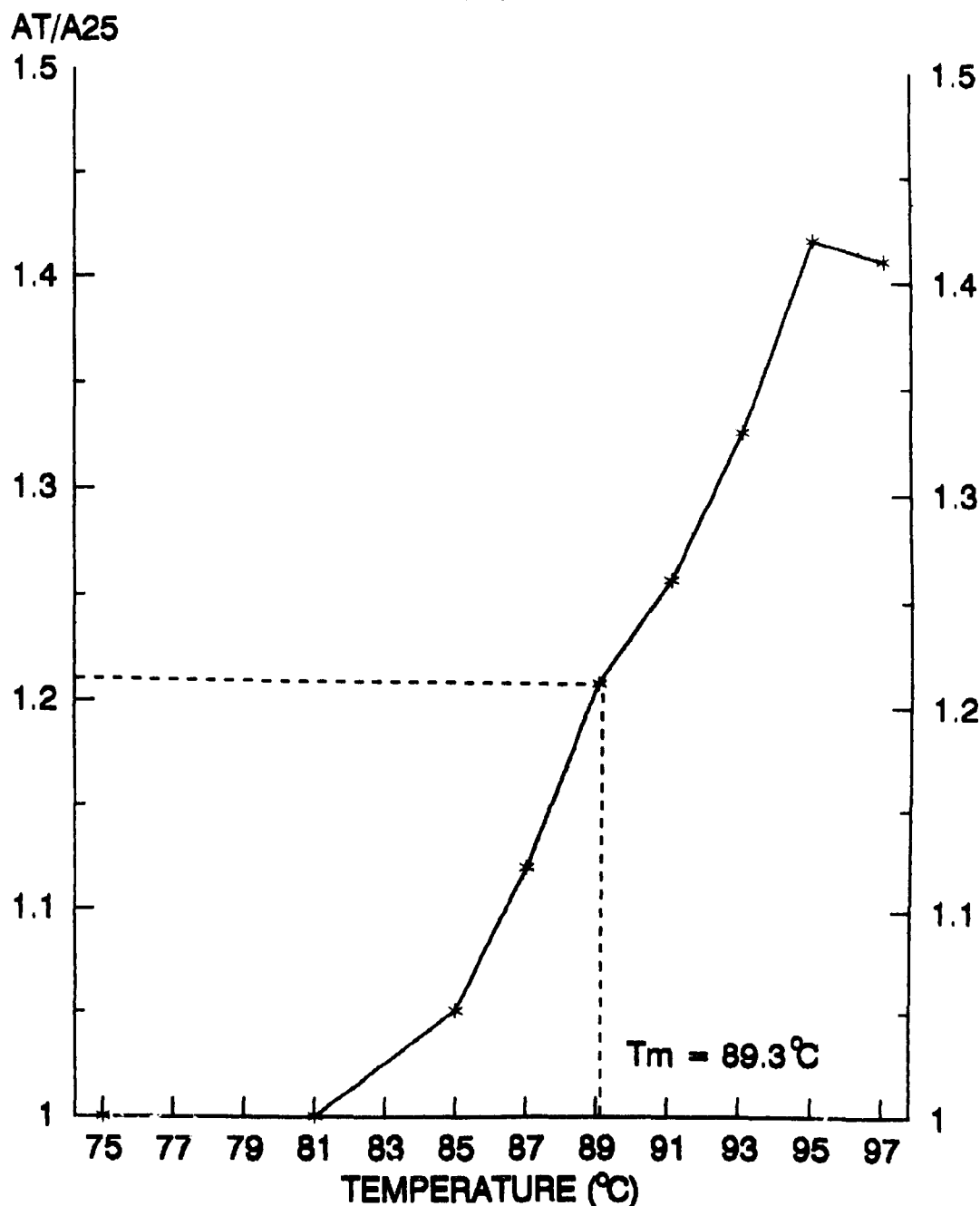
The low levels of activity seen with alanine dehydrogenase indicate that it is unlikely that alanine dehydrogenase plays any part in nitrogen assimilation in DR1 or IR1. Glutamate dehydrogenase in both IR1 and DR1 has low  $K_m$  values for ammonia whereas glutamine synthetase has a high  $K_m$  values for hydroxylamine in both bacteria. This indicates that glutamate dehydrogenase is likely to be the primary pathway of ammonia assimilation in both isolates. This is highly unusual, as the common path of assimilation of intracellular ammonia produced by nitrate reductase in most methanotrophs investigated is through GS/GOGAT and assimilation only proceeds through glutamine dehydrogenase when high concentrations of ammonia are present in the growth medium (Murrell & Dalton, 1983b). The results from the nitrogen assimilation enzyme assays point to an interesting phenomenon in these isolates. The presence of nitrogenase was not detected as there was no growth in the absence of any fixed nitrogen sources.

### 3.15 DNA thermal melting experiments

The thermal melting temperatures of DNA from both DR1 and IR1 were determined to calculate the DNA percentage G + C content. The procedure was also performed on Escherichia coli HB101 as a control. The melting curves are shown in fig. 3.13. The average value obtained for E. coli HB101 was 48.5%, slightly lower than the expected value of 50% but within the margin of error. IR1 was found to have an average value of 47% and DR1 had an average value of 49%. These values are more typical of the reported values for Type I methanotrophs of between 52-58 mol % G+C than the Type II methanotrophs (Green, 1991).

# Determination of mol% G+C

IR1



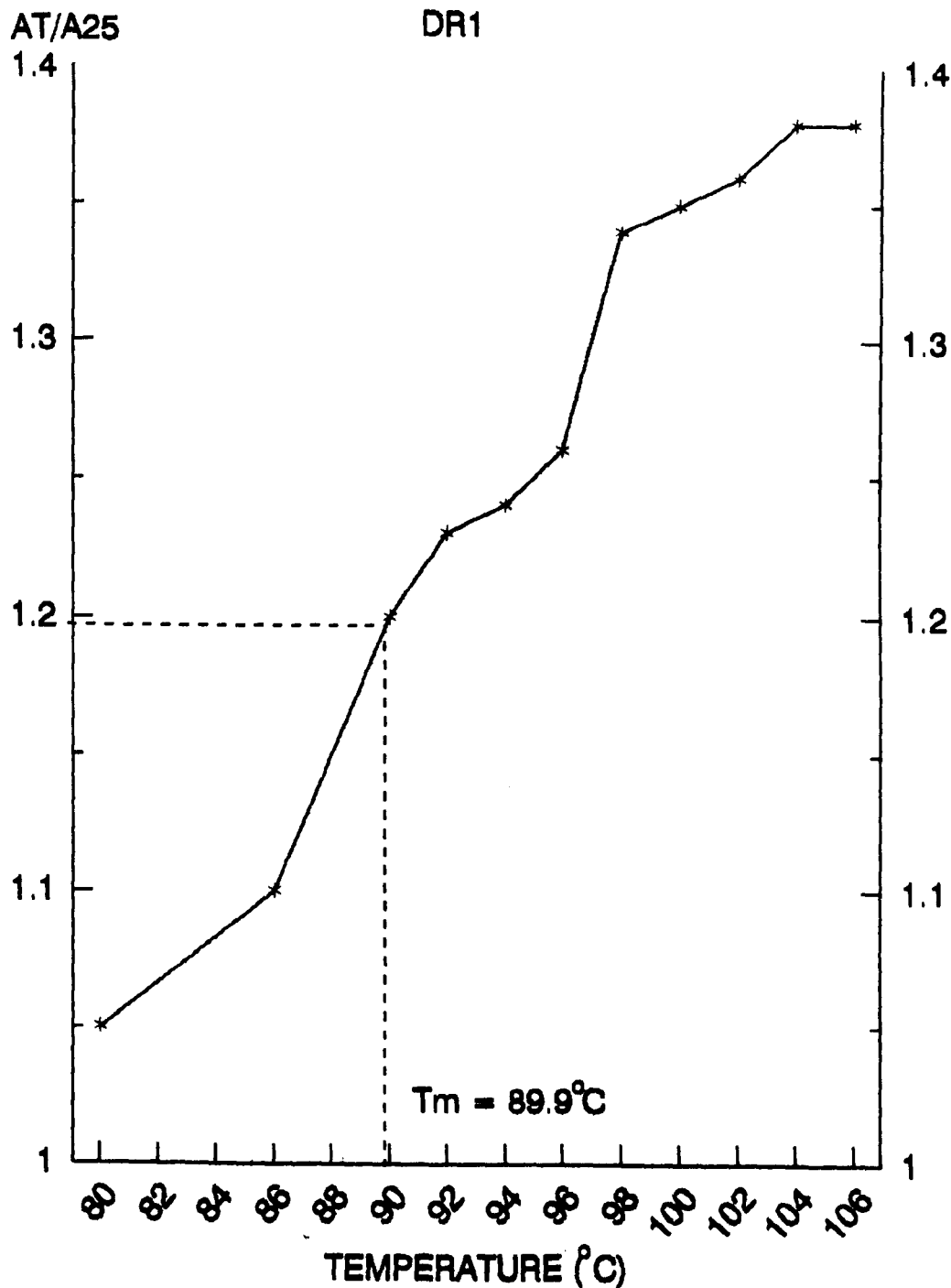
$$\begin{aligned}
 \text{mol\% G+C} &= (T_m - 69.3)2.44 \\
 &= (89.3 - 69.3)2.44 \\
 &= 48.8\%
 \end{aligned}$$

AT/A25 - ratio between absorbance at T°C (AT) and absorbance at 25°C (A25)

Tm - temp. at which  $AT/A25 = (\text{max. } AT/A25 - 1)/2$

*Fig. 3.13a* : Thermal denaturation profile of IR1

# Determination of mol% G+C DR1



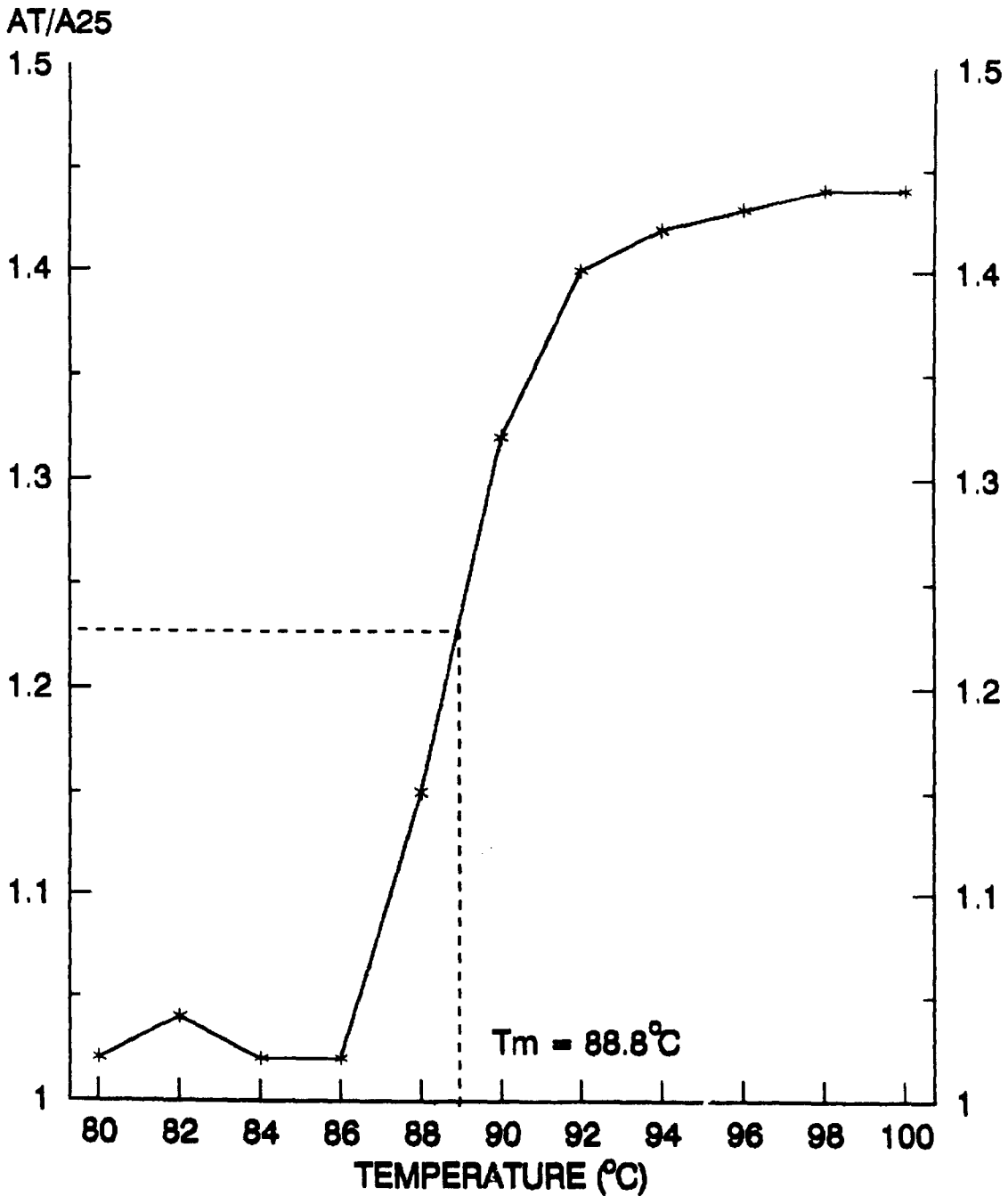
$$\begin{aligned}
 \text{mol\% G+C} &= (T_m - 69.3) 2.44 \\
 &= (89.9 - 69.3) 2.44 \\
 &= 50.3\%
 \end{aligned}$$

AT/A25 - ratio between absorbance at T°C (AT) and absorbance at 25°C (A25)

Tm - temp. at which  $AT/A25 = (\text{max. } AT/A25 - 1)/2$

**Fig. 3.13b** : Thermal denaturation profile of DR1

Determination of mol% G+C  
*Escherichia coli* HB101



$$\begin{aligned}
 \text{mol\% G+C} &= (T_m - 69.3)2.44 \\
 &= (88.8 - 69.3)2.44 \\
 &= 48\%
 \end{aligned}$$

AT/A25 - ratio between absorbance at T°C (AT) and  
absorbance at 25°C (A25)

Tm - temp. at which  $AT/A25 = (\text{max. } AT/A25 - 1)/2$

Fig. 3.13c Thermal denaturation profile of *Escherichia coli* HB101



### 3.16 Antibodies raised to IR1 and DR1

Initially, antibodies were raised to the isolates as a secondary method for detecting methanotrophs in the marine environment. However, due to the success of the techniques described in chapter 4, the antibodies were only preliminarily screened. Antibodies were raised to whole cells of IR1 and DR1 in rabbits. The serum which contained the antibodies was tested for specificity by Haem agglutination. Cells of both isolates were tested against both antibodies. Antibodies raised against IR1 showed no cross-reactivity to DR1 and vice versa. Reactivity of IR1 antibodies to IR1 cells, and DR1 antibodies to DR1 cells, showed a strong reaction. This showed that the antibodies were specific and could be developed as an aid to detection of similar organisms, but further screening is required.

### 3.17 Conclusions after enrichment and characterization experiments

Techniques for the isolation of marine methanotrophs have been developed. These methods gave rise to many methanotrophs from all environments tested. Marine methanotrophs appear to be sensitive to solid media, but whether this is due to toxicity of the solidifying agents used is unclear. A range of solidifying agents was tested and none was found to support growth of the marine methanotrophs isolated at concentrations normally used for plate culture work. It therefore appears that the most successful methods for isolation of marine methanotrophs at present are those which rely on liquid enrichment.

IR1 and DR1 were found to be Gram negative rods as described above. In common with all other characterized methanotrophs, they were

catalase positive and oxidase positive. Both isolates possessed a capsule and one or more flagella. The colonies appeared white in liquid culture and on soft agar plates, and orange/buff after centrifugation. They showed no growth on any solid media, but did form colonies on soft agar plates formed by the addition of 0.4% Noble agar. Neither isolate showed true microaerophilic growth, although they demonstrated a slight sensitivity to oxygen. No pigment was formed in low-iron concentrations, as is the case in Methylocystis cultures. In common with most verifiable reports, IR1 and DR1 had a limited carbon substrate range and could only utilize methane and methanol. No growth was observed on any other single carbon compound, or on more complex organic carbons. Neither isolate showed any ability to fix  $N_2$ . Both could utilize potassium nitrate but could not grow on ammonium chloride as a sole nitrogen source, even though it appeared that ammonium chloride was not toxic to the cultures. A range of amino acids were suitable as sole nitrogen sources. Both IR1 and DR1 required sodium chloride for growth, although this could be substituted to a certain extent by lithium chloride, sodium fluoride and calcium chloride. The growth optima for temperature, sodium chloride and pH were determined. For both IR1 and DR1 the optimum temperature for growth was 30°C and the optimum pH value was 7.6. The pH value was higher than that observed in other methanotrophs, but as sea water has pH values between 7.2 and 7.8, this is not unexpected. The sodium chloride tolerance of both isolates was broad, and was similar to that seen in other marine species (Sieburth et al, 1987). Carbon assimilation into the cell from formaldehyde proceeds via the ribulose monophosphate pathway, which is typical of Type I methanotrophs (Green,

1991). The classification of IR1 and DR1 into Type I organisms is supported by the observation that they did not appear to possess a soluble methane monooxygenase and that they had %mol G+C contents of approximately 49%. Nitrogen assimilation is unusual in IR1 and DR1, as they appeared to assimilate nitrogen into the cell from ammonia to glutamate by glutamate dehydrogenase, and not by the more common pathway of GS/GOGAT.

It appears that the two new marine methanotrophic isolates are typical of other Type I methanotrophs with several differences. Firstly, they have an absolute requirement for sodium chloride, and are halotolerant, unlike any other methanotroph tested. They also appear to assimilate nitrogen by an unusual route. This may reflect the environment in which they occur naturally. The isolates most resemble Methylomonas species and are similar to the described characteristics of Methylomonas pelagica isolated by Sieburth et al, 1987.

## DEVELOPMENT OF DETECTION TECHNIQUES

#### 4.1 Detection of marine methylotrophs

The results of the enrichment experiments described in section 3 indicated that methanotrophs in the marine environment were difficult to culture by conventional techniques. The marine methanotrophs isolated appeared to be sensitive to solid media and liquid enrichments gave rise to few distinct methanotroph strains. There are many recent reports describing the problems faced by microbiologists who have attempted the detection and enumeration of bacterial groups purely by cultivation of the bacteria (for example, Pickup, 1991). It was therefore considered that methods for detecting marine methanotrophs which did not rely on their cultivation should be developed. These are essential if an understanding of the occurrence and quantity of methanotrophs in the marine environment is to be attempted.

The development of a detection method was approached by exploiting molecular biology techniques to selectively detect genes encoding key enzymes present in the bacteria. Two such key enzymes present in methylotrophs and, more specifically, methanotrophs, are methane monooxygenase and methanol dehydrogenase. Methane monooxygenase (MMO) is unique to methane-oxidizing bacteria, whereas methanol dehydrogenase (MDH) is found in all bacteria with the ability to oxidize methanol, including the methanotrophs. There have been many studies performed on these enzymes and the gene sequence of the soluble MMO (sMMO) gene cluster from two methanotrophs has been determined [Methylococcus capsulatus (Bath) (Stainthorpe et al, 1989; 1990a) and Methylosinus trichosporium (OB3b) (Cardy et al, 1990)]. The gene sequence of the structural gene of MDH has also been determined in the methylotrophs Methylobacterium extorquens AM1 (Anderson et al,

1990); Methylobacterium organophilum XX (Machlin & Hanson, 1988) and Paracoccus denitrificans (Harms et al, 1987). There is close homology between the two sMMO sequences and also between the three MDH sequences. This indicated that detection of sMMO and MDH genes in DNA isolated from environmental samples could offer a method for detecting methanotrophs and methylotrophs. In order to detect the small quantity of homologous DNA in an environmental sample, it was necessary to adapt procedures for amplification of specific DNA sequences. Polymerase chain reaction (PCR) methods offered amplification techniques which had been well established in clinical microbiology and were beginning to be applied in environmental detection (see section 1.7.9 for further details).

It was decided to develop detection techniques which would allow detection of both methylotrophs and methanotrophs. As MDH is present in all Gram negative methylotrophs, amplification of MDH-specific sequences is not specific for methanotrophs. However, whilst all methanotrophs possess MMO in a particulate form (pMMO), only some also possess sMMO. Although pMMO gene probes would provide a specific probe for all methanotrophs, it has so far proved impossible to isolate the pMMO protein and therefore the sequence of the gene encoding that protein.

## 4.2 Methanol dehydrogenase-specific amplification

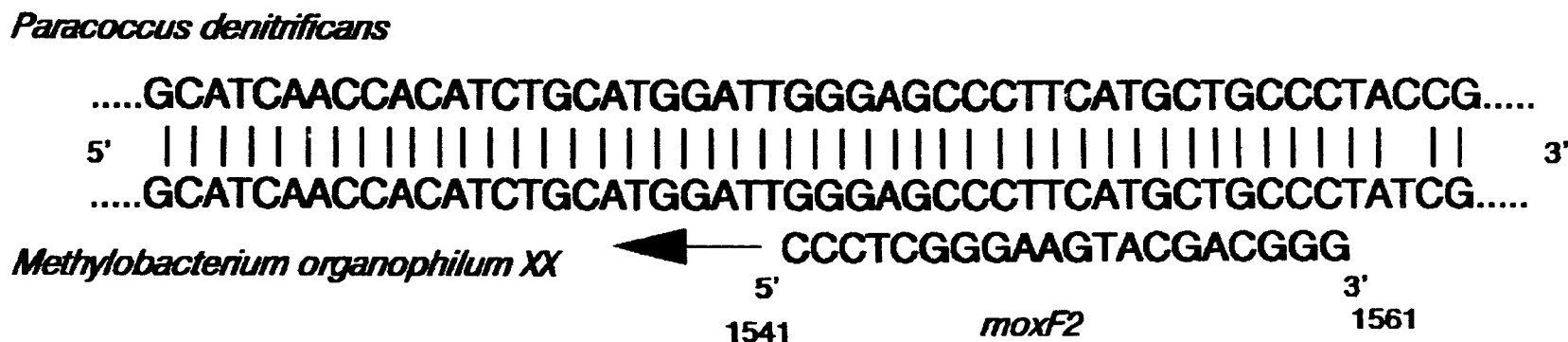
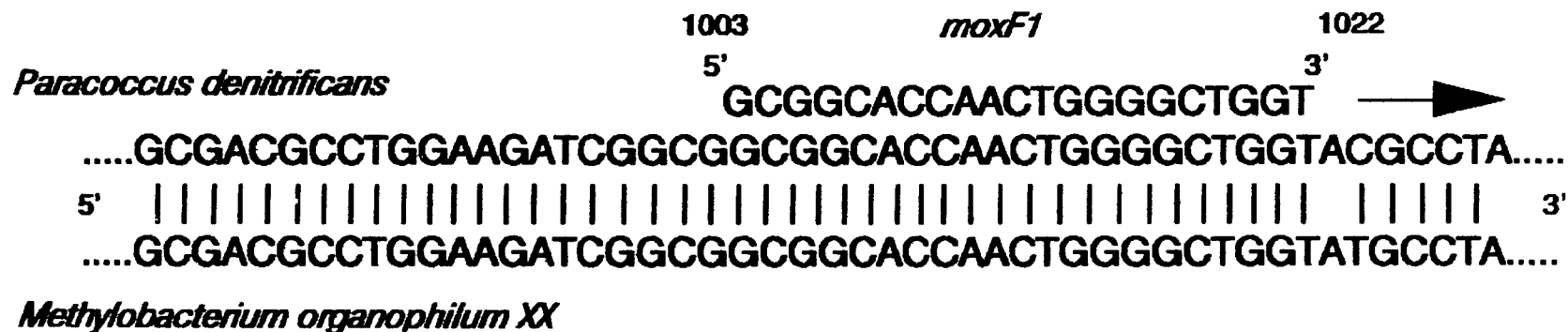
### 4.2.1 Development of primers for amplification of MDH-specific sequences

The three published sequences for the structural gene of MDH, moxF, were analyzed to compare regions of homology between all three

sequences. Two regions which showed extensive conservation between the species were selected. The melting temperature (that temperature at which 50% of the DNA solution is denatured and 50% is annealed) was determined by the G + C content of the regions. It was important that the melting temperature of both primers were similar to allow the primers to anneal to the template at low temperatures and separate at higher temperatures. Two primers each of 20 base pairs length were chosen and are illustrated in fig. 4.1. The primers were approximately 550 base pairs apart from each other. P<sub>moxF1</sub> (priming 5' to 3') was 75% G + C and P<sub>moxF2</sub> (which primed 3' to 5') was 70% G + C. This gave a theoretical melting temperature of 72°C for P<sub>moxF1</sub> and 68°C for P<sub>moxF2</sub>. The sequences chosen did not contain any regions which could allow the primers to self-anneal in preference to the template. The moxF gene from Methylobacterium extorquens AM1 was also available on a plasmid pMOXFG (Nunn & Lidstrom, 1986) together with moxG, moxI and moxJ genes as shown in figure 4.2. The moxF gene was excised from this plasmid by digestion with XhoI. The resulting fragments were separated by agarose gel electrophoresis and a 3 Kb fragment containing the moxF gene was excised from the gel. The DNA was then purified by the 'Gene-clean' method. After radio-labelling the gene was used as a methylotroph specific probe.

#### 4.2.2 Amplification of MDH specific sequences

Initially, PCR reactions were set up in the following buffer



**Fig. 4.1 : PCR primers designed to be specific to the MDH structural gene.**  
Base pairs refer to P. denitrificans gene.



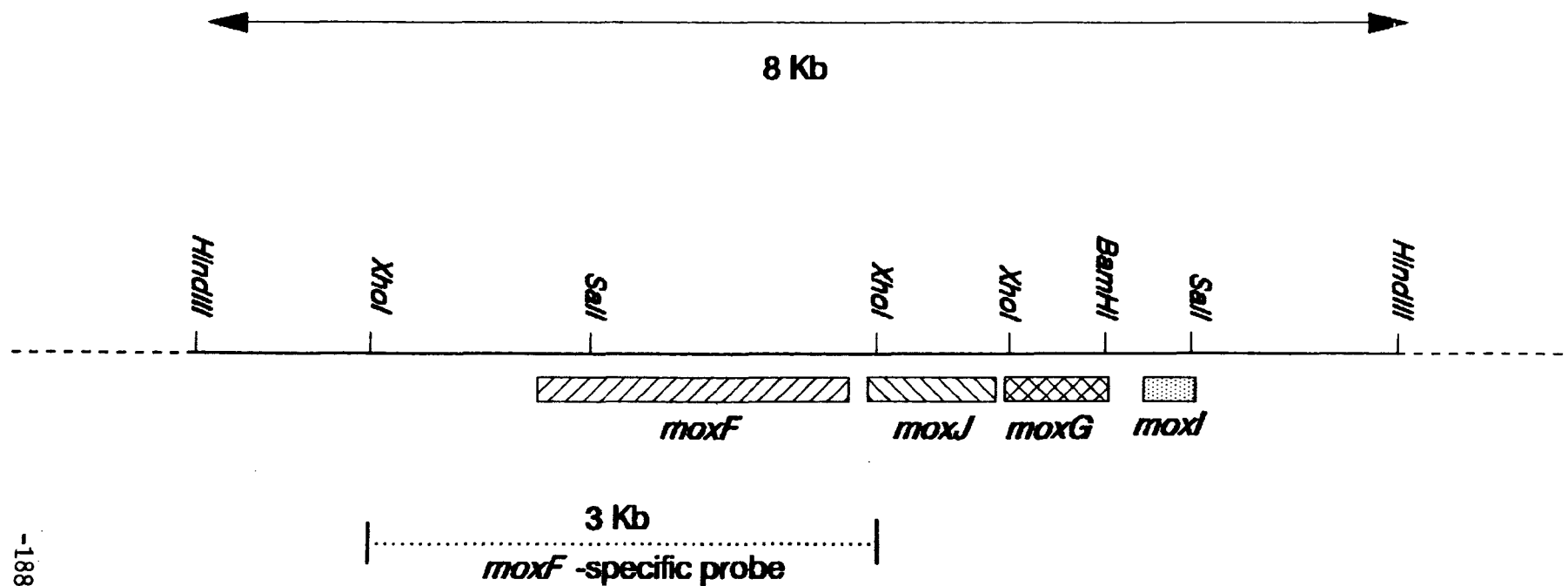


Fig.4.2 : Restriction map of plasmid pMOXFG, containing *moxF* *moxJ* *moxG* and *moxI* genes

----- pVK100 DNA

..... probe used to identify *moxF*-specific DNA

40 mM Tris pH 8.3  
5 mM MgCl<sub>2</sub>  
70 mM KCl  
430  $\mu$ M DTT  
420  $\mu$ M each dNTP  
30 pM each primer  
20 ng/ $\mu$ l sheared salmon sperm DNA  
1-2 units Taq polymerase

All reactions were placed into a Perkin Elmer Cetus thermocycler. The reactions were cycled through the following for 40 cycles:

denature 94°C 1 min  
anneal 55°C 1 min  
extend 72°C 1 min

A number of DNA samples were used as templates in the reactions to amplify MDH sequences. These are shown below:

Mediterranean sea water DNA  
Mediterranean sea water DNA spiked with M. trichosporium (OB3b)  
Methylosinus trichosporium (OB3b) DNA

No amplification was observed in any of these samples.

A PCR kit from Perkin Elmer Cetus was then purchased. This contained test primers with template DNA as a control, and commercially prepared buffer and dNTPs. It also contained a Taq polymerase produced by genetic engineering called 'Amplitaq'. Buffer, dNTPs and 'Amplitaq' from the kit together with MDH-specific primers and template DNA from Methylosinus trichosporium (OB3b) were used to amplify MDH-specific sequences within the DNA. As a control to ensure that the amplification reactions were proceeding correctly, test template from the PCR kit was amplified with the primers provided. The reactions were carried out on a Hybaid combi thermocycler and cycled through the following for 40 cycles:

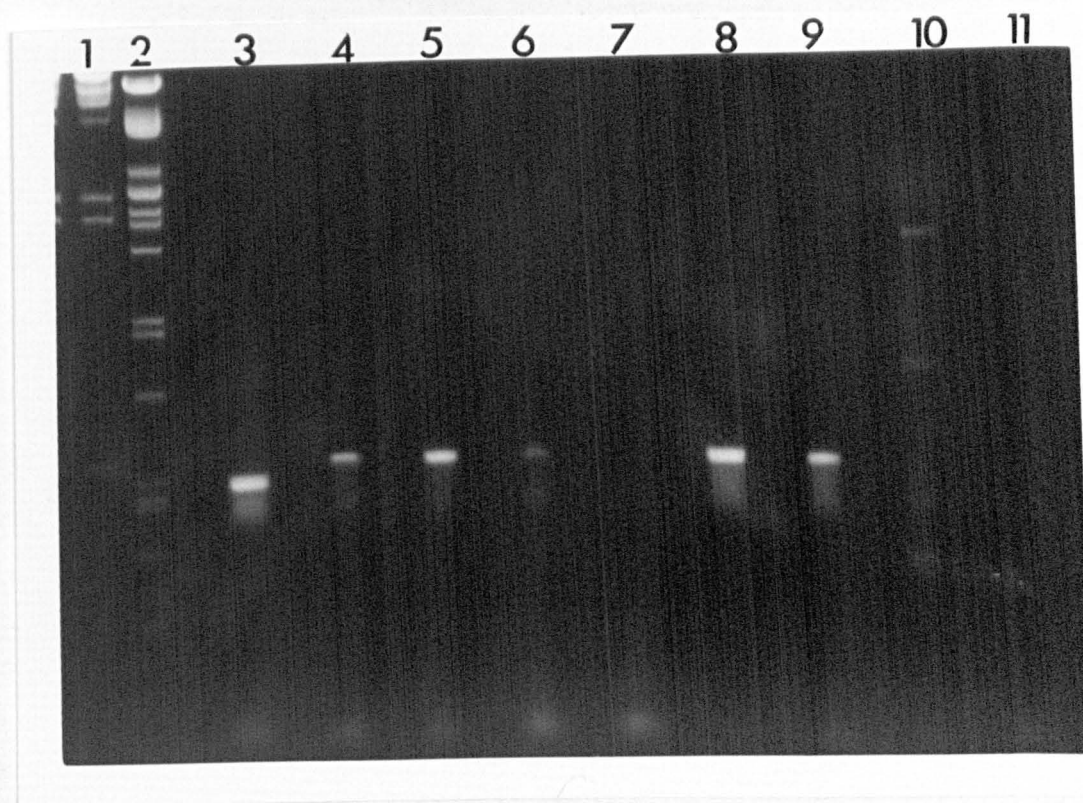
denature 94°C 1min

anneal 37°C 1 min

extend 72°C 1 min

After amplification a PCR product of approximately 550 bases was produced (fig 4.3). After subsequent hybridization with a radio-labelled moxF gene from Methylobacterium extorquens AM1 this was confirmed to be a MDH-specific product. Pure DNA from Methylosinus trichosporium (OB3b) was subsequently used as a positive control whenever PCR was performed. DNA from Escherichia coli HB101 was used as a negative control and was not amplified when MDH-specific primers were used.

DNA was extracted from both marine and fresh water sites and was obtained by the method of Fuhrman et al (1987). 10 ml methanotroph culture (with an absorbance at 540 nm of approximately 1.0) were added



- |   |   |
|---|---|
| 1: $\lambda$ HindIII markers                        | 7: DNA extracted from Tocil Lake                      |
| 2: $\lambda$ PstI markers                           | 8: DNA extracted from Mediterranean (spiked with IR1) |
| 3: PCR control                                      | 9: DNA extracted from Mediterranean                   |
| 4: OB3b   | 10: HB101   |
| 5: IR1  | 11: negative control (no DNA)                         |
| 6: DNA extracted from Tocil Lake (spiked with OB3b) |   |

**Fig. 4.3** Agarose gel electrophoresis of PCR products from amplification of methanol dehydrogenase-specific sequences.

to some of the water samples before filtration of the sample and extraction of DNA. DNA thus 'spiked' was then used as positive controls. The areas examined and the samples which were spiked are shown in table 4.1. An example of the quality and quantity of DNA obtained from the extraction method used is shown in fig. 4.4. DNA from the areas described was used as a template for PCR using the MDH-specific primers. The products obtained were separated by agarose gel electrophoresis and the results are shown in fig. 4.5. It was observed that a positive result produced a 550 base pair fragment and that there were few or no non-specific bands. All areas tested appeared to yield DNA which when PCR-amplified gave a product of the correct size except DNA from a water sample from NAC (Stoneleigh) pond. This pond was known to be anaerobic, and it was thought it unlikely to contain methylotrophs.

#### 4.2.3 Confirmation that the PCR products were MDH-specific

The products described above were transferred from the agarose gels onto nitrocellulose filters by Southern blotting and hybridized with the moxF gene from Methylobacterium extorquens AM1. This confirmed that the products obtained were indeed methylotroph-specific. The hybridization results are shown in fig. 4.6.

#### 4.2.4 Sequencing of PCR products

The products obtained after PCR using pMOXFG and DNA from the marine isolate IR1 as templates were sequenced to confirm the PCR product from the reactions was MDH and to compare the sequences with known MDH sequences. The products were sequenced by a direct

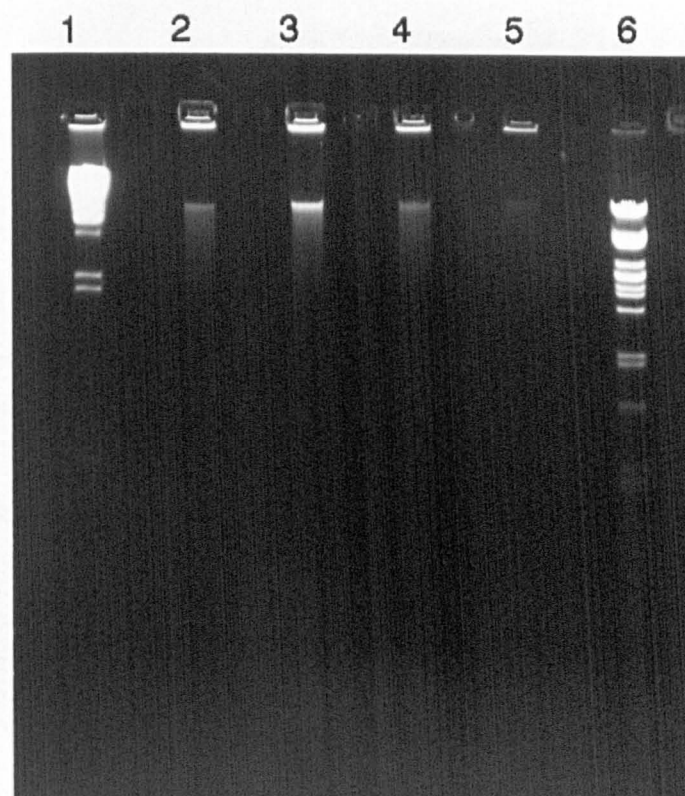
ENVIRONMENTAL DNA SAMPLES
SEA WATER
North Atlantic Plymouth Sound Mediterranean North Sea
FRESH WATER
Chesford Grange pond, Leamington Spa Newbold Comyn Golf course pond, Leamington Spa Tocil lake, University of Warwick East Site pond, University of Warwick River Leam, Leamington Spa Grand Union Canal, Leamington Spa Stoneleigh pond, N.A.C., Stoneleigh

**Table 4.1:** Environmental DNA sampling sites.

NON-METHANOTROPHIC CULTURES
<i>Escherichia coli</i> HB101 <i>Myxococcus xanthus</i> <i>Klebsiella</i> <i>Erwinia caratovora</i> subsp. caratovora <i>Streptomyces lividans</i> <i>Synechococcus</i> DC2 (cyanobacterium) <i>Methylobacterium extorquens</i> AM1 (methylotroph) IR1 } (methanotrophs containing pMMO, DR1 } but not sMMO)

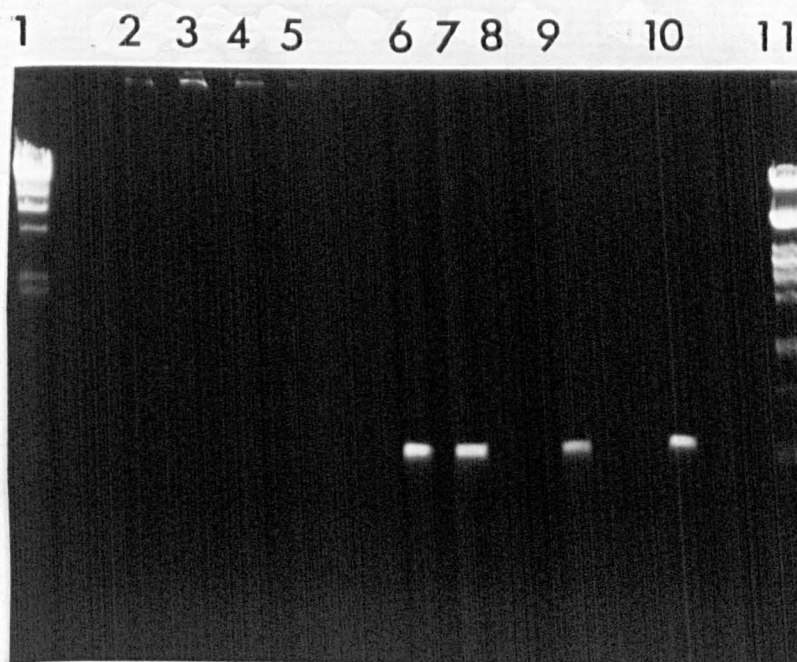
**Table 4.2:** Non-methanotrophic cultures used to examine the specificity of sMMO and MDH amplification.

MMO - methane monooxygenase  
(p - particulate; s - soluble)  
MDH - methanol dehydrogenase



- 1:  $\lambda$ /HindIII markers
- 2: River Leam
- 3: Grand Union Canal
- 4: NAC, Stoneleigh
- 5: Chesford Grange
- 6:  $\lambda$ /PstI markers

*Fig. 4.4 : DNA obtained from environmental samples.*

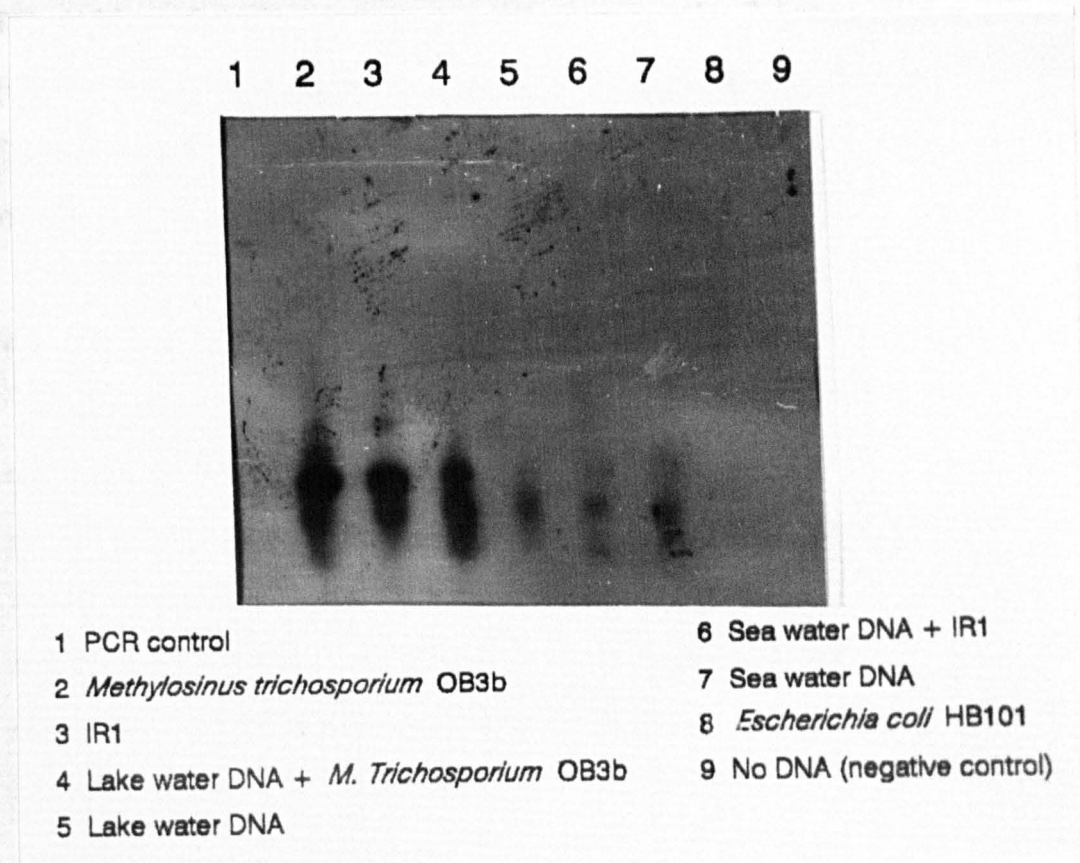


- 1:  $\lambda$ /HindIII markers
- centrifugation*
- 2: River Leam
- 3: Grand Union Canal
- 4: NAC, Stoneleigh
- 5: Chesford Grange
- filtration*
- 6: River Leam
- 7: Grand Union Canal
- 8: NAC, Stoneleigh
- 9: Chesford Grange
- 10: IR1 control
- 11:  $\lambda$ /PstI markers

*Fig. 4.5: Results of amplification of environmental DNA with methanol dehydrogenase-specific primers. DNA was extracted by two methods: centrifugation and filtration - see text for details*



sequencing method (see materials and methods). The results are shown in Fig 4.7. The sequence from pMOXG differed from the published sequence data of *moxF* from *Methylobacterium* strains by the bases over a 200 base pair length. This is due to the inaccuracy of the polymerase, which has a typical error rate of 12. The sequence of



**Fig. 4.6 :** DNA extracted from pure methanotrophic cultures and from environmental samples PCR-amplified with *moxF*-specific primers and hybridized to  $^{32}\text{P}$ -labelled *moxF* fragment.

The agarose gel in fig. 4.3 was blotted onto nitrocellulose. After hybridization, the filter was washed at  $60^\circ\text{C}$  in 2 x SSC.



sequencing method (see materials and methods). The results are shown in fig 4.7. The sequence from pMOXFG differed from the published sequence data of moxF from Methylobacterium extorquens AM1 by two bases over a 200 base pair length. This is due to the infidelity of Taq polymerase, which has a reported error rate of 1%. The sequence of IR1 differs from M. extorquens AM1 sequence by 7 bases over a 100 base pair length. Although some of the error will be due to misreading by Taq polymerase, the sequence of MDH in IR1 is expected to be slightly different to that of M. extorquens AM1.

#### 4.2.5 Comparison between PCR detection and conventional cultivation

Environmental samples which yielded DNA found to produce an MDH-specific product after PCR amplification were tested for the presence of methanotrophic bacteria by conventional techniques. Water from Leam river; Newbold Comyn pond; the canal at Leamington Spa; NAC, Stoneleigh pond; Chesford Grange pond; Tocil lake and Mediterranean was used for this investigation (see table 4.1). 1 l water was used to set up enrichment cultures for methane oxidizers. All the cultures were turbid after 5 days of incubation with the exception of the sample from NAC, Stoneleigh. Microscopic analysis of turbid cultures revealed the presence of methanotrophs. Subsequent sub-culture of the enrichment cultures proved that the turbidity was due to methane utilization and not to traces of organics in the water samples. As mentioned before, the water from NAC, Stoneleigh was known to be anaerobic and therefore was unlikely to contain any methanotrophic or methylotrophic bacteria, confirming that results obtained by PCR were supported by conventional cultivation techniques.

*Methylobacterium extorquens* AM1  
pmoxfg PCR product

10	20	30	40	50
ACCGGCAACC	CGGCGCCGTG	GAACGAGACC	ATGCTGCCGG	GCGACAACAA
ACCGGCAACC	CGGCGCCGTG	GAACGAGACC	ATGCTGCCGG	GCGACAACAA
60	70	80	90	100
GTGGACGATG	ACGATCTTCG	GCCGCGATGC	CGATACGGGT	GAAGCCAAGT
GTGGACGATG	ACGATCTTCG	GCCGCGATGC	CGATACGGGT	GAAGCCAAGT
110	120	130	140	150
TCGGCTACCA	GAAGACCCCG	CACGACGAGT	GGGACTATGC	CGGCGTCAAC
TCGGCTACCA	GAAGACCCCG	CACGACGAGT	GGGACTATGC	CGGCGTCAAC
160	170	180	190	
GTCATGATGC	TCTCCGCGCA	GAAGGACAAG	GACGGCAAGG	CCCGC
	*			
GTCATGATGC	TCTCCGAGCA	GAAGGACAAG	GACGGCAAGG	CCCGC

Fig. 4.7a: Homology between the gene sequence of *Methylobacterium extorquens* AM1 and the PCR product obtained after amplification of pMOXFG with MDH-specific primers.

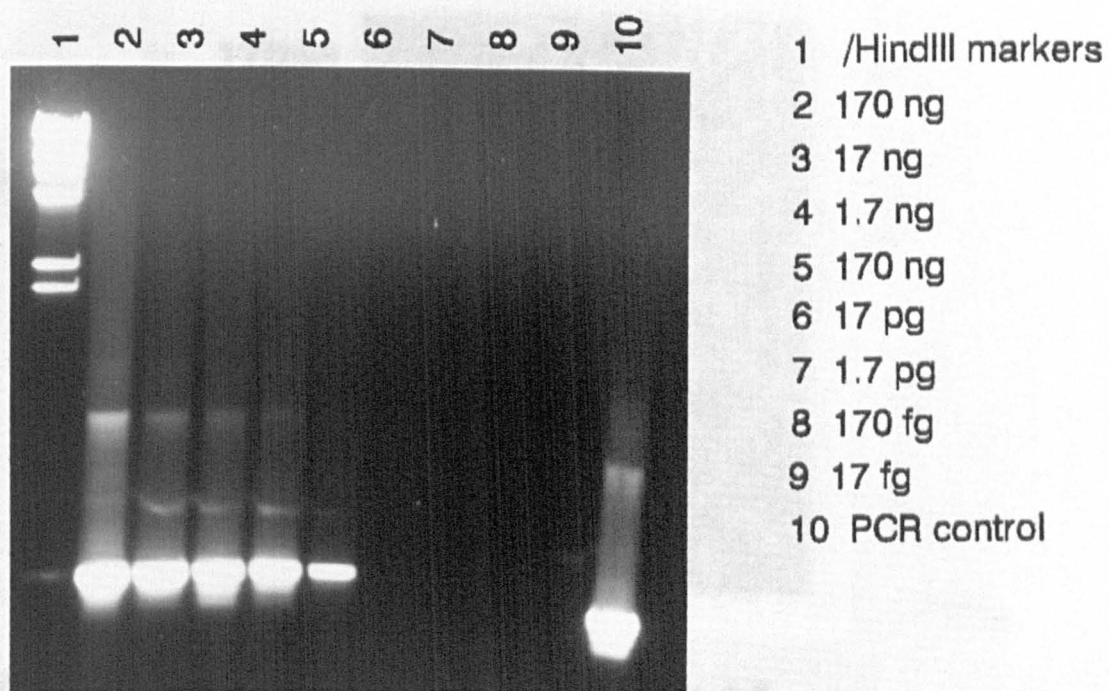
*Methylobacterium organophilum* XX  
IR1 PCR product

10	20	30	40	50
.....	.....TG	GAACGAGACC	ATGCGTCCCG	GCGACAACAA
		*		
.....	.....TG	GAACGAAACC	ATGCGTCCCG	GCGACAACAA
60	70	80		
GTGGACGATG	ACGATCTTCG	GCCGCGAC		
* * * *	* * *	* * *		
ATGGACCATG	ACCATCTGGG	GACGCGAC		

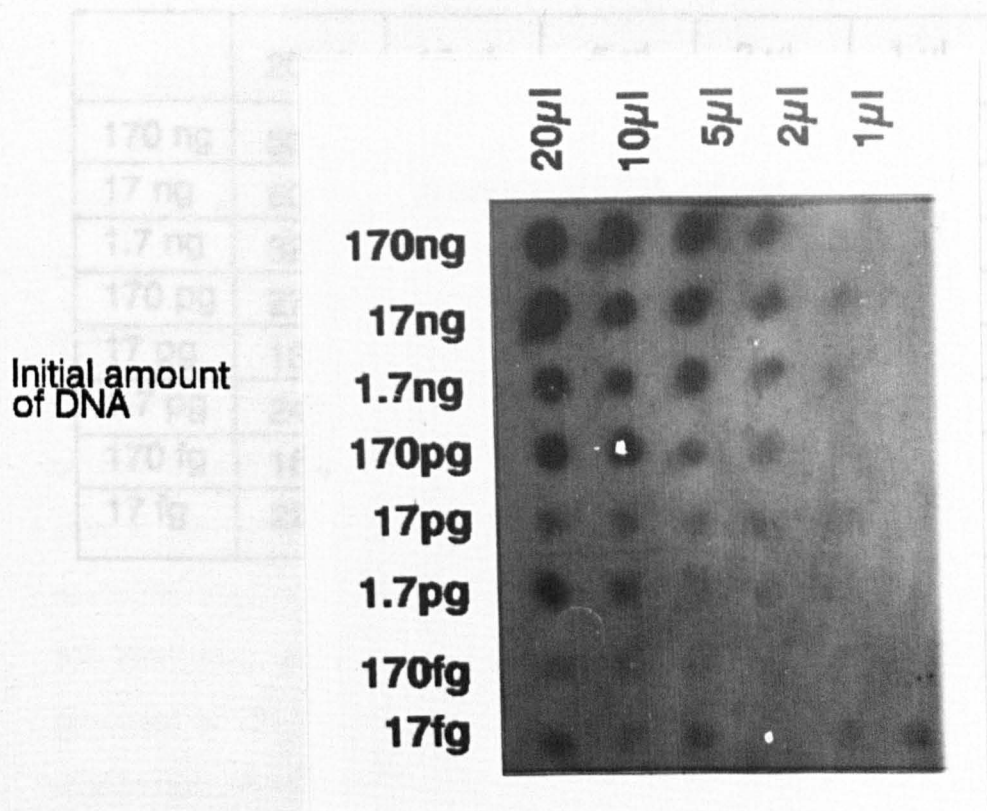
Fig. 4.7b: Homology between the gene sequence of *Methylobacterium organophilum* XX and the PCR product obtained after amplification of marine isolate IR1 by MDH-specific primers.

#### 4.2.6 Enumeration of methylotrophs by PCR

In order to use PCR as a method for enumerating methylotrophs, the amount of MDH-specific sequences in the unamplified sample has to be assumed from the final amplified product. This is possible with the following constraints: firstly, control reactions containing known quantities of methylotroph DNA need to be carefully processed. Any error with these reactions can complicate subsequent comparison with environmental controls. Secondly, only relatively 'clean' DNA can be quantified. Environmental DNA from soil or heavily polluted waters, for example, contain substances known to inhibit PCR. DNA samples from water taken from unpolluted lakes and rivers and from sea water does not inhibit PCR to a great degree. Initial experiments were performed to test that different quantities of template DNA could be distinguished after amplification. A dilution series was made from a known amount of DNA and amplified. 5  $\mu$ l of the resulting products were separated on an agarose gel and are shown in fig. 4.8. The remaining reaction mixture was spotted onto nitrocellulose by using a dot-blot manifold and hybridized to radiolabelled moxF gene probe from pMOXFG (fig. 4.9). Finally, the nitrocellulose filter was cut into squares, with one 'dot' in each square. The radioactivity of each square was calculated by scintillation counting. It was assumed that the greater the amount of product, the more radiolabelled probe would have bound. The results are shown in fig. 4.10. It can be seen that for greater amounts of template DNA, more product is formed and more radiolabelled probe binds. The results obtained here indicate that enumeration of methylotrophs in aquatic samples by PCR may be possible. It must be emphasised, however, that for enumeration to be possible, the dilution



*Fig. 4.8:* PCR-amplification by *moxF*-specific primers of DNA dilution series. DNA extracted from isolate IR1 was serially diluted before amplification.



**Fig. 4.9 : Dot Blot of dilution series shown in Fig. 4.8.**  
 Filter hybridized with  $^{32}\text{P}$ -labelled *moxF* fragment. After hybridization the filter was washed at  $55^{\circ}\text{C}$  in  $2 \times \text{SSC}$ .

	20 $\mu$ l	10 $\mu$ l	5 $\mu$ l	2 $\mu$ l	1 $\mu$ l
170 ng	533.8	433.8	290.2	233.4	126.6
17 ng	504.7	251.3	167.5	135.6	130.6
1.7 ng	321.4	196.4	170.5	138.6	110.7
170 pg	272.3	171.5	135.6	136.6	104.7
17 pg	131.2	145.6	96.7	121.7	109.7
1.7 pg	241.3	117.7	88.7	76.8	94.7
170 fg	169.2	101.7	67.8	63.8	115.7
17 fg	221.3	130.6	125.6	133.6	143.6

**Fig. 4.10 : Scintillation counter results from enumeration series.**  
**DNA dilution series (after PCR-amplification) hybridized to *max F***  
**After exposure to autoradiographic film, the filter was cut into**  
**squares containing one spot/square. The radioactivity of each**  
**square was calculated by scintillation counting.**  
**At lower levels, the measurements become less accurate,**  
**but when 20  $\mu$ l reaction mix was loaded, the results are**  
**accurate to 170 fg target DNA.**

series performed above must be repeated for each set of amplification reactions.

#### 4.2.7 Ensuring MDH PCR product is methylotrophic specific

It had been shown that the product from amplification by PCR using MDH specific primers gave rise to a product that hybridized to the moxF gene of Methylobacterium extorquens A1. It was necessary to confirm that this product was unique to methylotrophs and was not produced by non-methylotrophic bacteria. DNA from pure cultures of a number of methylotrophs and several non-methylotrophic bacteria were tested for amplification by MDH-specific primers. The results are shown in fig. 4.11. All methylotrophic DNA samples tested produced a 550 base pair product. None of the non-methylotrophic DNA samples produced a 550 base pair product. In some of the samples, bands which are larger than 550 base pairs can be observed. These are due to non-specific binding of the primers and are easily distinguished from the positive product by the intensity on agarose gels and their size. The samples were loaded onto nitrocellulose by a Dot-Blot manifold and hybridized with the moxF gene (fig. 4.12). A clear signal was observed from the methylotrophic samples, whilst no signal was seen from the non-methylotrophic samples, indicating that the moxF primers amplified DNA specific to methylotrophs.

#### 4.2.8 Comparison of thermocyclers

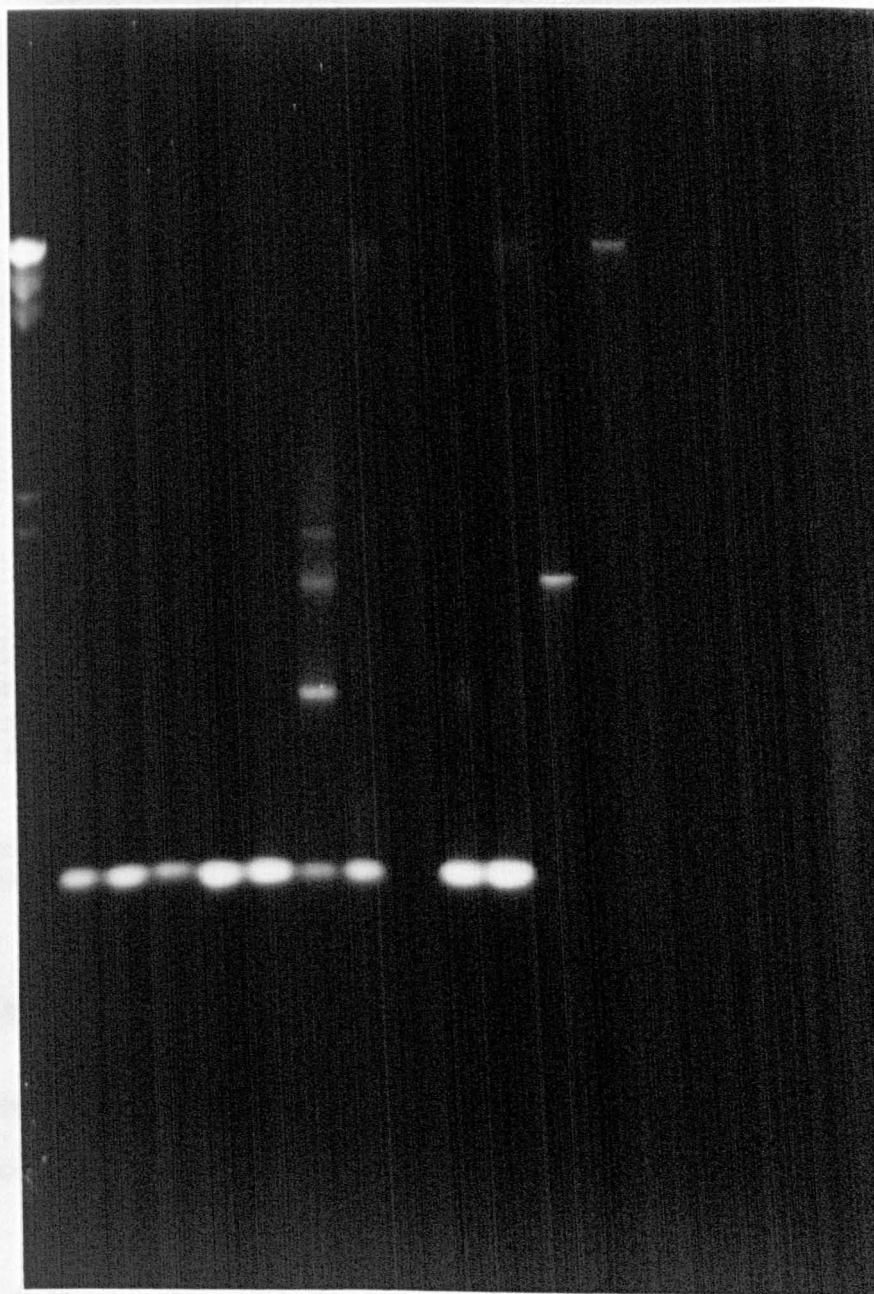
As part of the optimization of PCR techniques, several different thermocyclers were investigated. These are listed below:

*Fig. 4.11: Specificity of moxF primers. DNA from a number of bacteria was used as template DNA. A 500 bp product was produced by DNA from methanotrophs and methylotrophs, but not by DNA from non-methylotrophic bacteria.*

Track    1: *Methylococcus capsulatus* (Bath)  
          2: *Methylosinus trichosporium* OB3b  
          3: Marine isolate IR1  
          4: Marine isolate DR1  
          5: *Methylobacteria capsulatus* Y  
          6: *Methylobacterium extorquens* AM1  
          7: *Methylomonas methanica* A4  
          8: *Methylomonas agile* A20  
          9: *Methylosinus trichosporium* PG  
        10: plasmid pMOXFG  
        11: *Xanthobacter*  
        12: *Shigella*  
        13: *Synechococcus* strain DC2  
        14: *Klebsiella*  
        15: *Escherichia coli* HB101  
        16: *Streptomyces lividans*  
        17: *Myxococcus xanthus*  
        18: *Erwinia caratovora*

DNA was obtained from culture collection stocks at the University of Warwick.





1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

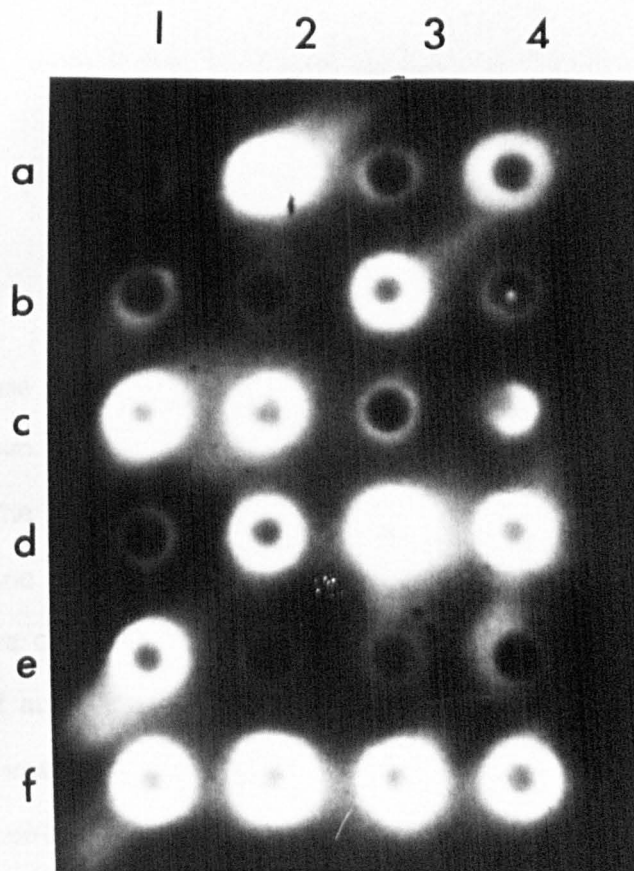


Fig. 4.12: Dot-blot of PCR reactions shown in fig. 4.11. 20  $\mu$ l was spotted onto nitrocellulose filter and probed with *moxF* fragment. After hybridization, the filter was washed at 55°C in 2xSSC. Full bacterial names are given in fig. 4.11.

1a: *Erwinia*  
 1b: *Streptococcus*  
 1c: A4  
 1d: DC2  
 1e: AM1  
 1f: pMOXFG

2a: OBBP  
 2b: *Shigella*  
 2c: PG  
 2d: OB3b  
 2e: *E. coli* DH1  
 2f: MCB

3a: *Klebsiella*  
 3b: BG8  
 3c: *Myxococcus*  
 3d: S1  
 3e: BC1  
 3f: M2\*

4a: IR1  
 4b: *Synechocystis*  
 4c: 5  
 4d: Y  
 4e: HB101  
 4f: DR1

\* MSA-utilizer isolated by S. Baker (Pers. comm.)

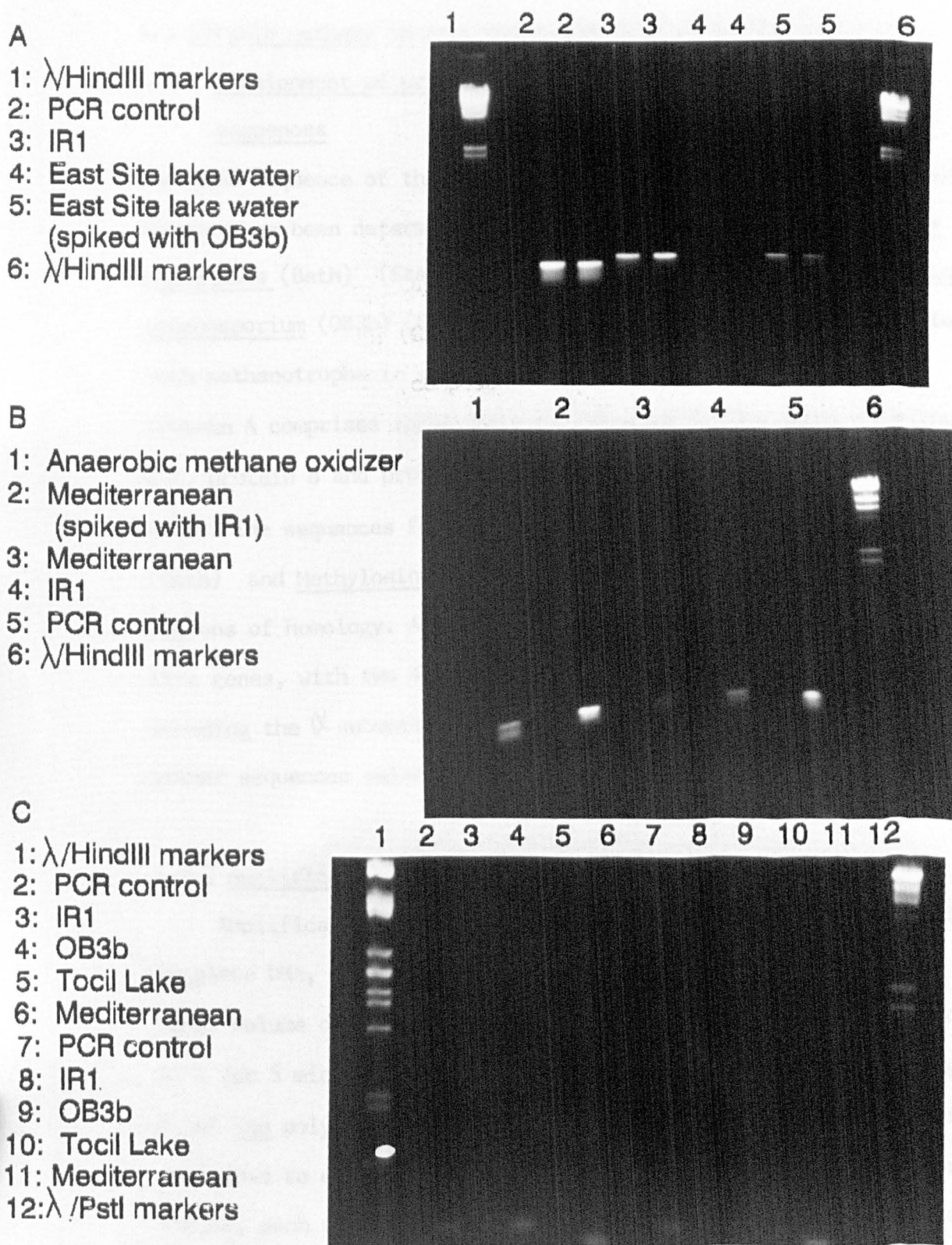
Techne PHC-3

LEP

Perkin Elmer Cetus

Hybaid Combi TR2

The same reactions and conditions were used in all four machines and are shown below. A comparison of the results obtained is shown in fig 4.13. The product obtained after amplification in the Perkin Elmer Cetus machine was clean, with no double banding. The temperature changes were quick and accurate. The Hybaid machine, which is halogen lamp heated and air cooled, gave similar results. The temperature change was very rapid, although the machine cannot cool to 4°C. The timing was not started until the machine was within 1°C of the target temperature. The Techne machine gave amplification, although the product band was a doublet. The temperature change was slow, and often inaccurate. The timing started when the temperature was within 4°C of the target temperature. The length of time of heating increased during the programme and often the machine did not reach the annealing temperature. The amplified product from the LEP machine was again a doublet. This machine actually heated quickly, but overshot the target temperature, and cooled back down before starting the timing, which resulted in a longer time at high temperatures than that programmed. The conclusion from this is that all machines tested were adequate for diagnostic PCR. For reproducibility, however, the Hybaid Combi and the Perkin Elmer Cetus machines proved to be more reliable. On price, the Hybaid Combi was one third of the published price for Perkin Elmer Cetus.



*Fig. 4.13: Comparison of thermocyclers.*

A: Perkin Elmer Cetus

B: LEP

C: Techne

Amplification by Hybaid machine demonstrated in Fig. 4.3.

### 4.3 Soluble methane monooxygenase-specific amplification

#### 4.3.1 Development of primers for amplification of sMMO-specific sequences

The gene sequence of the soluble methane monooxygenase (sMMO) gene cluster has been determined for two methanotrophs [Methylococcus capsulatus (Bath) (Stainthorpe et al, 1989; 1990) and Methylosinus trichosporium (OB3b) (Cardy et al, 1991a; 1991b)]. The sMMO complex of both methanotrophs is composed of three components; A, B and C.

Protein A comprises three subunits,  $\alpha$ ,  $\beta$  and  $\gamma$ . The genes encoding  $\alpha$ ,  $\beta$  &  $\gamma$ , protein B and protein C are found within the gene cluster (fig. 4.14). The sequences for the genes for both Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) were analyzed to compare regions of homology. A set of two primers were chosen for each of the five genes, with two sets of primers chosen for mmoX (the gene encoding the  $\alpha$  subunit). The homology between the sequences and the primer sequences selected are illustrated in fig. 4.15 and 4.16.

#### 4.3.2 Amplification of MMO-specific sequences

Amplification of MMO-specific sequences was performed by adding template DNA, primers, nucleotides and Taq polymerase buffer to a final volume of 50  $\mu$ l into an Eppendorf tube and placing the tube at 94°C for 5 minutes to denature the template DNA. After annealing, 0.3 $\mu$ l Taq polymerase was added to each reaction, and 50  $\mu$ l parafin oil was added to each tube. The tubes were then placed into the thermocycler, each reaction heated to 72°C for 1 min for extension and cycled through the following for 40 cycles:

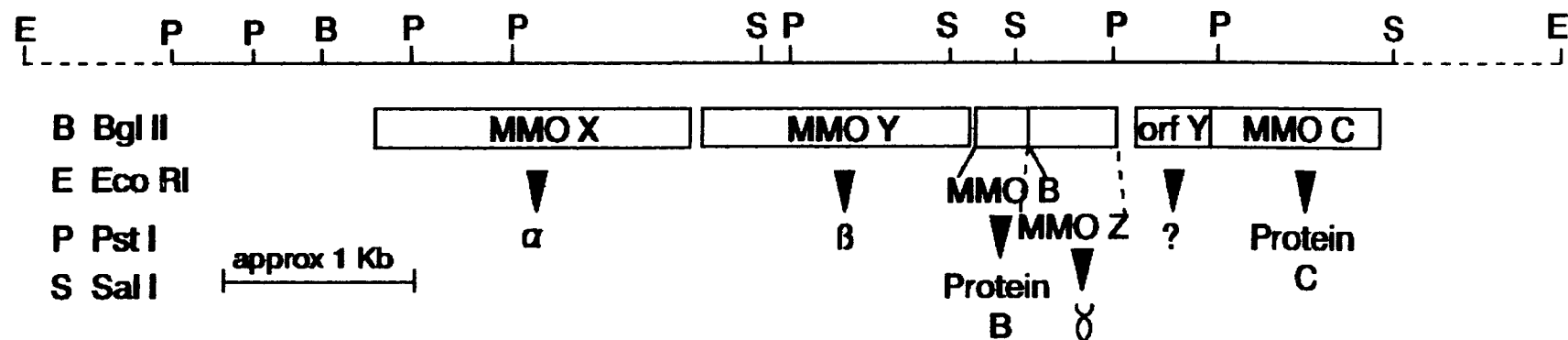


Fig. 4.14 : Soluble methane monooxygenase gene cluster in *Methylococcus capsulatus* (Bath)

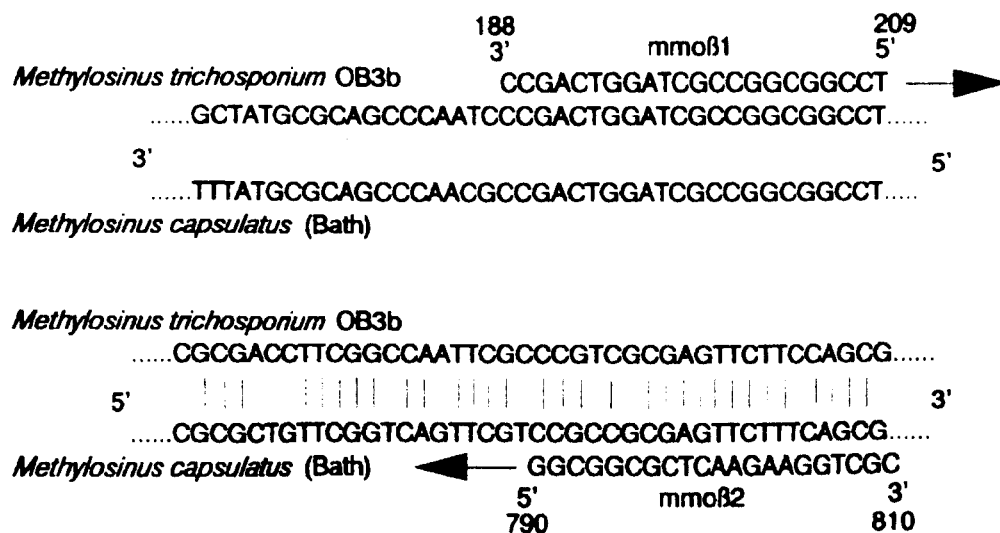


Fig. 4.15a : PCR primers designed to be specific to the gene encoding the  $\beta$ -subunit of sMMO

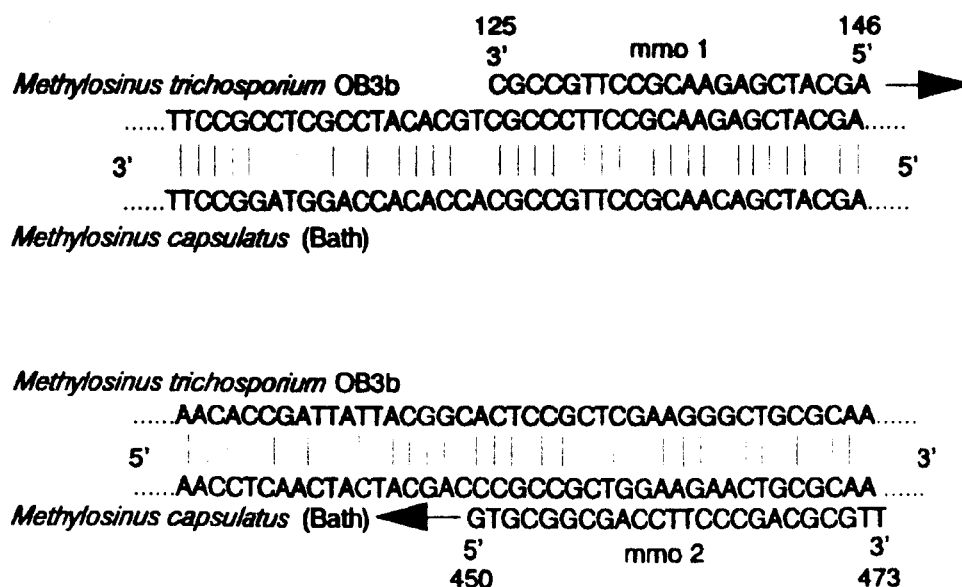


Fig. 4.15b PCR primers designed to be specific to the gene encoding the  $\alpha$ -subunit of sMMO



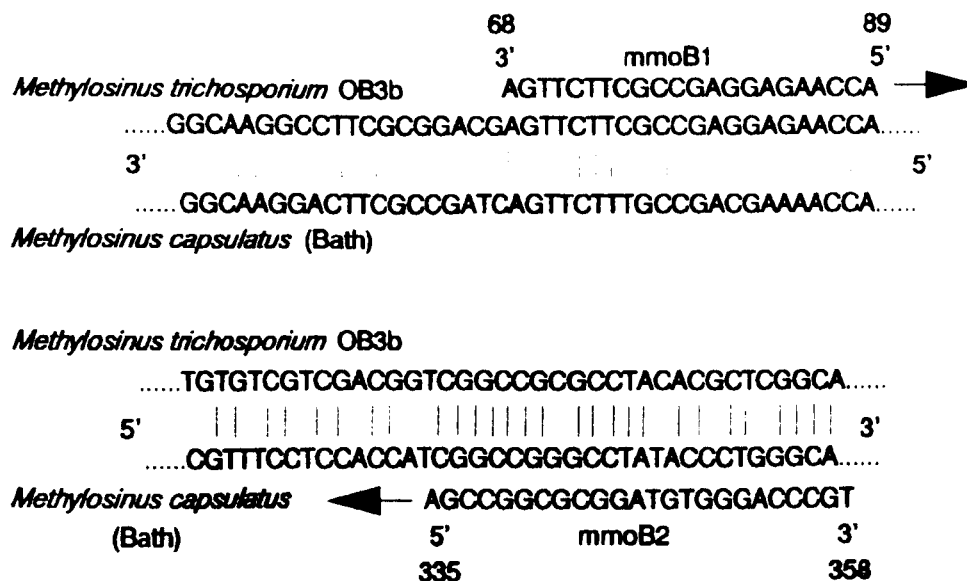


Fig. 4.15c PCR primers designed to be specific for the gene encoding protein B of sMMO

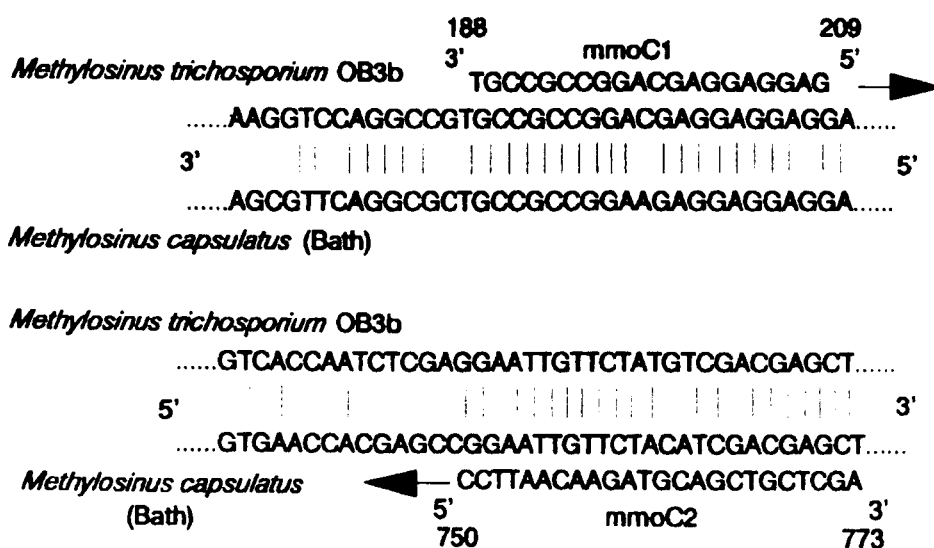
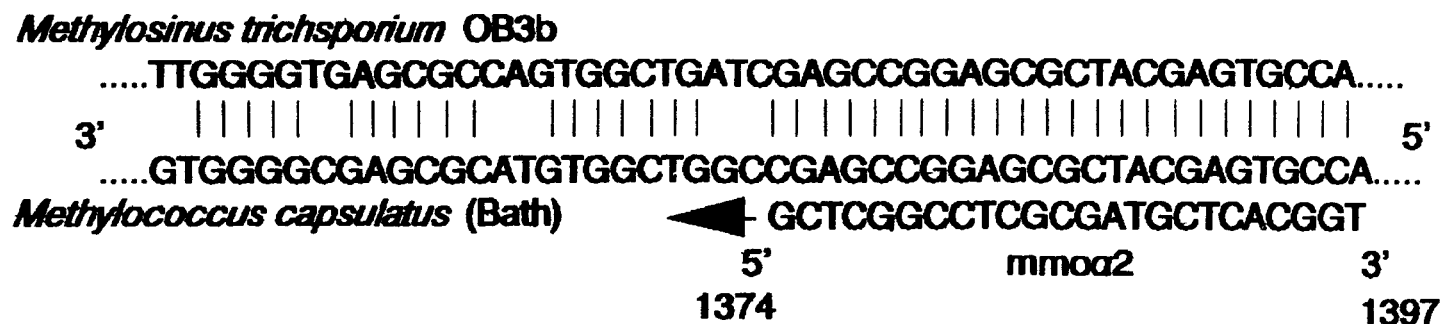
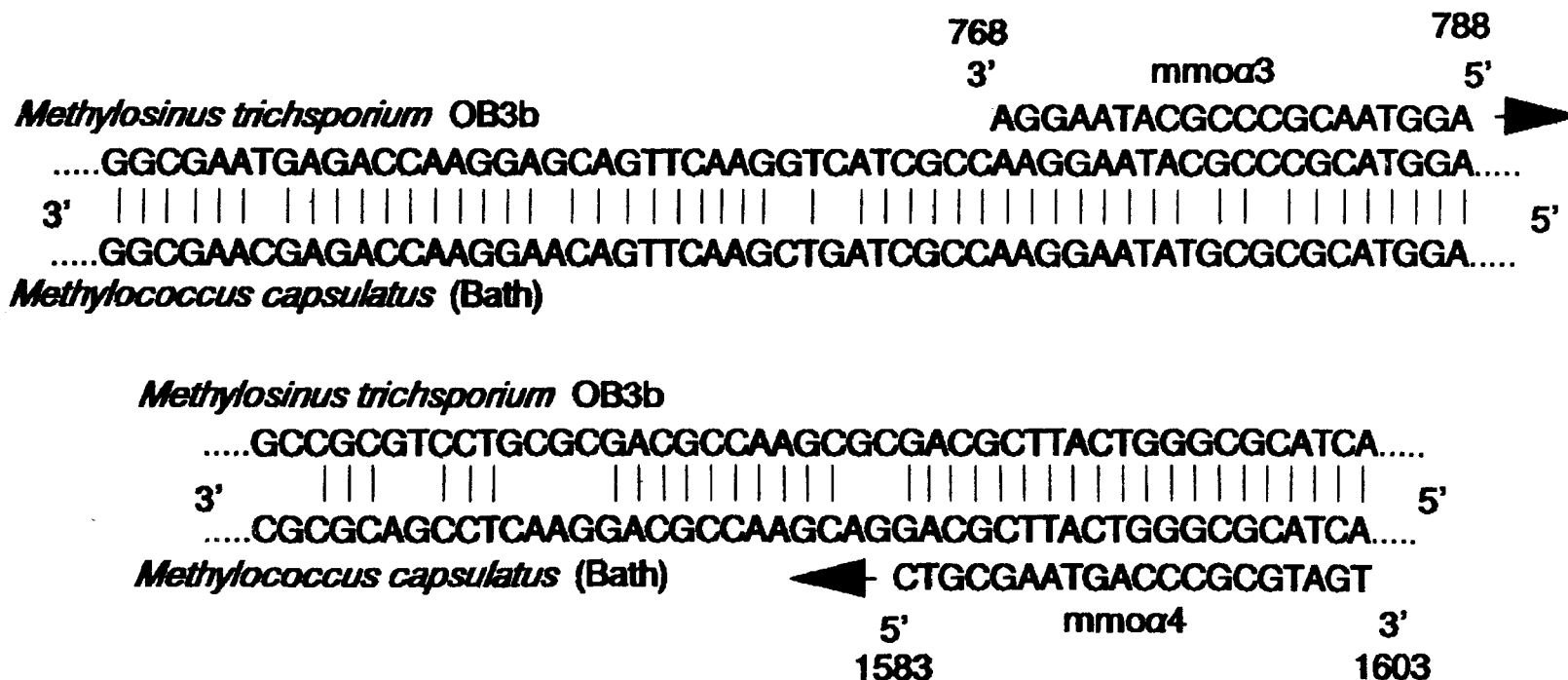


Fig. 4.15d PCR primers designed to be specific to the gene encoding protein C of sMMO





**Fig. 4.16a** : PCR primers designed to be specific to the gene encoding the  $\alpha$ -subunit of sMMO. The primers were chosen to be outside the region showing homology to ribonucleotide reductase (see text for details).



**Fig. 4.16b :** PCR primers designed to be specific to the gene encoding the  $\alpha$ -subunit of sMMO. The primers were chosen to be outside the region showing homology to ribonucleotide reductase (see text for details).

denature 1 min

anneal 1 min

extend 1 min

The temperature of denaturing and extension were as described for MDH-specific sequence amplification. The annealing temperature was determined for each set of primers and is discussed below.

#### 4.3.3 Amplification of *mmoX* - the $\mu$ subunit

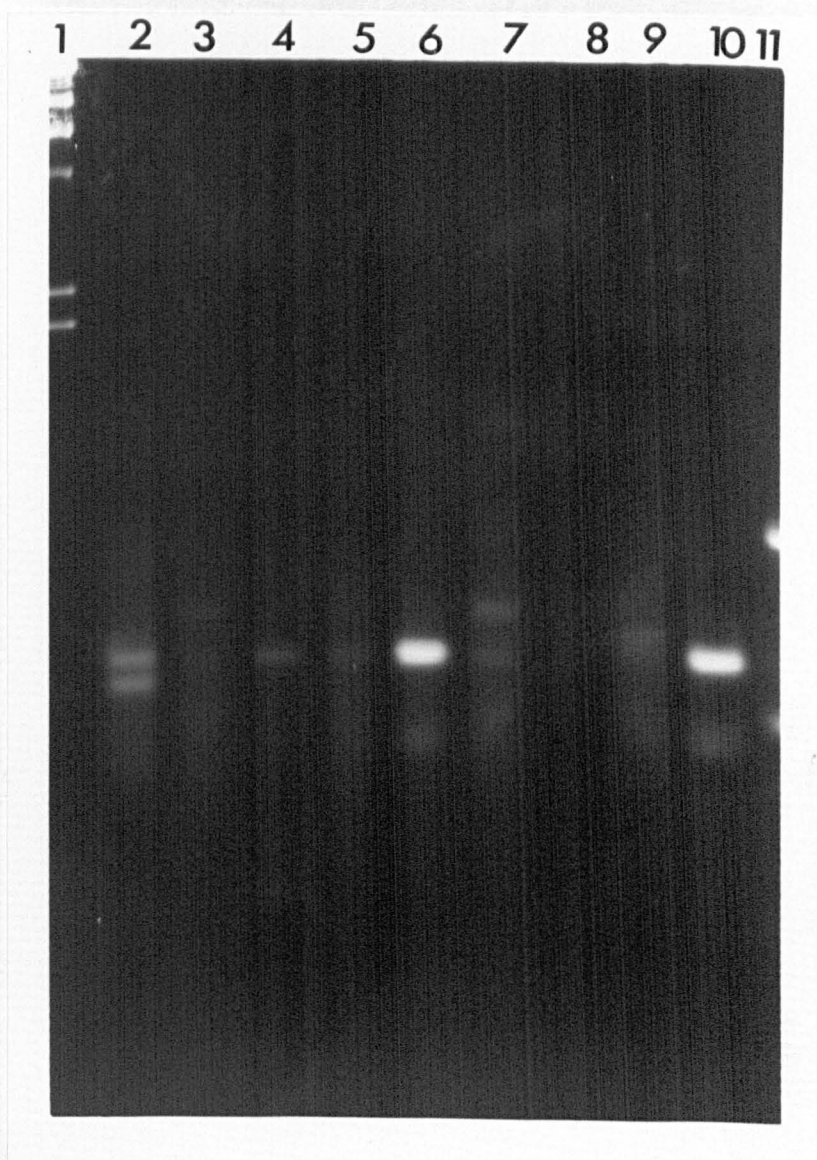
mmoX encodes the  $\mu$  subunit of protein A from SMO. The iron-centre active site of SMO is likely to reside on this subunit (Prior & Dalton, 1985). The region that codes for the  $\mu$ -hydroxy bridge iron centre has homology with a number of other iron-centre proteins (Stainthorpe et al, 1990) and care was taken in ensuring that the primers chosen to amplify mmoX did not occur within this region. Two sets of primers were chosen for amplifying mmoX. The first set spanned the putative 'active site' region, whilst the second set occurred well beyond it (fig. 4.16). After amplification, a product of 1.5 Kb was expected to be produced by the first set of primers and 535 bp by the second. Amplification of mmoX was attempted with different templates for both primers and the annealing temperature was 37°C.

Amplification using both sets of primers was achieved when pCH4, the plasmid containing the SMO region of Methylococcus capsulatus (Bath), was used as a template. No amplification, however, was seen when chromosomal DNA purified from Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) was used as a template. This was surprising. The mmoX gene shows good homology between Methylococcus

capsulatus (Bath) and Methylosinus trichosporium (OB3b) (94% at the amino acid level) and if there is amplification of the plasmid DNA, there is no apparent reason why the chromosomal DNA should not have been amplified.

#### 4.3.4 Amplification of *mnoY* - the $\beta$ subunit

mnoY encodes the  $\beta$  subunit of protein A from sMMO. The homology between the mnoY of Methylococcus capsulatus (Bath) 1989) and that of Methylosinus trichosporium (OB3b) was found to be 83.5% at the amino acid level (Cardy et al, 1991a). Two primers were selected to amplify mnoY, which would give a product after amplification of 602 bp. Initial amplification was performed at an annealing temperature of 37°C, but subsequent experiments indicated that the stringency of amplification could be increased and the annealing temperature was raised to 55°C. Amplification of both Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) produced PCR products of the correct size. A range of environmental DNA samples (as listed in table 4.1) were amplified using mnoY-specific primers and also produced PCR products of the correct size (fig. 4.17). To confirm that the PCR products were mnoY-specific, Southern blots and dot-blot of the PCR products were hybridized with a radiolabelled probe for mnoY (see fig. 4.18). This gave a positive signal, even under conditions of high stringency. To confirm that the primers were specific for methanotrophs, a range of non-methanotrophic bacterial DNAs were amplified (listed in table 4.2) (fig. 4.19). There was no amplification of these DNAs using the mnoY-specific primers and no hybridization with the mnoY probe. To confirm that DNA from the environment did not contain



1:  $\lambda$ HindIII markers  
 2: North Atlantic  
 3: Plymouth Sound  
 4: North Sea  
 5: River Leam

6: Chesford Grange pond  
 7: Newbold Cornyn pond  
 8: East Site pond  
 9: Tocil Lake  
 10: MCB  
 11: HB101

**Fig. 4.17** Results of amplification of the gene encoding the  $\beta$ -subunit  
*(mmoY)*

Fig. 4.18: PCR products amplified with  $\beta$ -specific primers and hybridized with mmoY DNA probe.

(DNA extracted from Tocil Lake)

Track 1: 2.5  $\mu$ l each primer  
2: 1.25  $\mu$ l each primer  
3: 0.6  $\mu$ l each primer  
4: 0.4  $\mu$ l each primer  
5: 0.2  $\mu$ l each primer

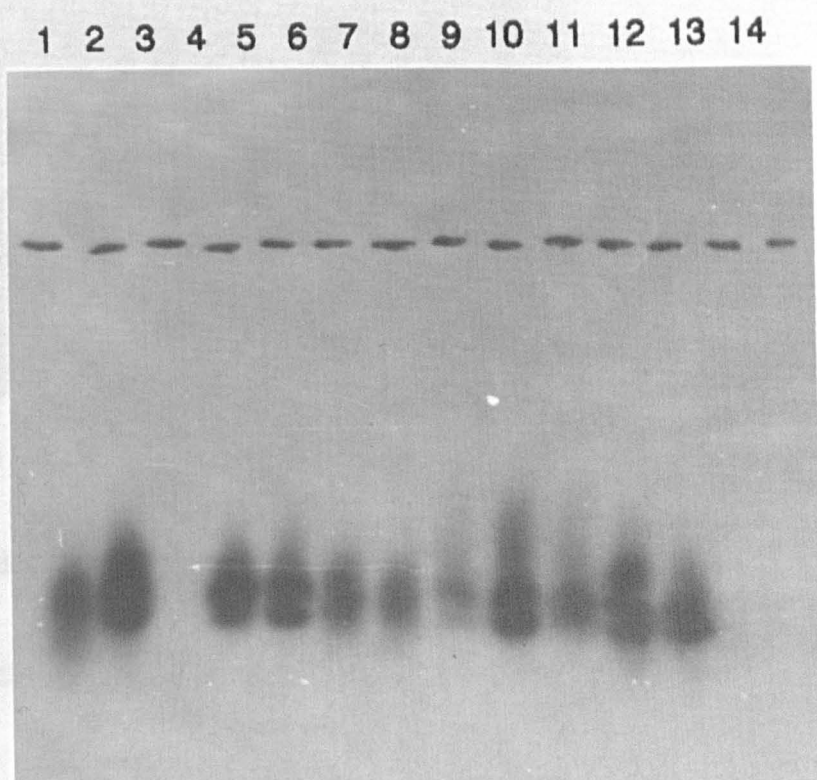
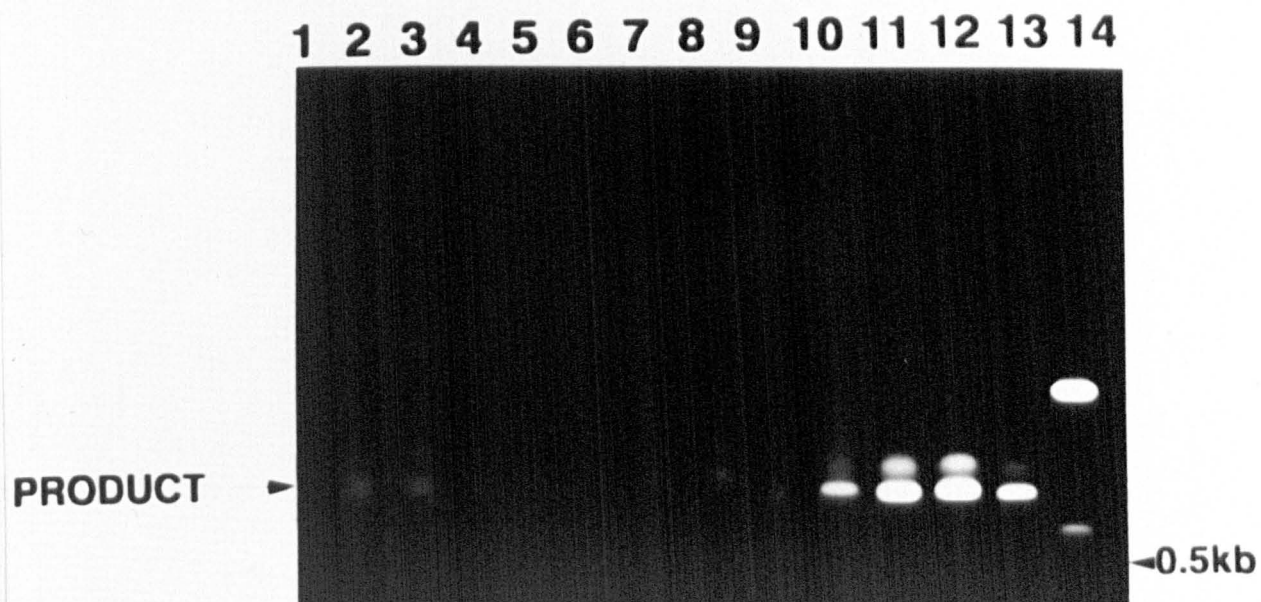
(DNA extracted from East Site pond)

Track 6: 0.4  $\mu$ l each primer  
7: spiked with  $10^4$  *M.capsulatus* cells ml<sup>-1</sup>  
8: spiked with  $10^3$  *M.capsulatus* cells ml<sup>-1</sup>  
9: spiked with  $10^2$  *M.capsulatus* cells ml<sup>-1</sup>  
10: spiked with  $10^1$  *M.capsulatus* cells ml<sup>-1</sup>

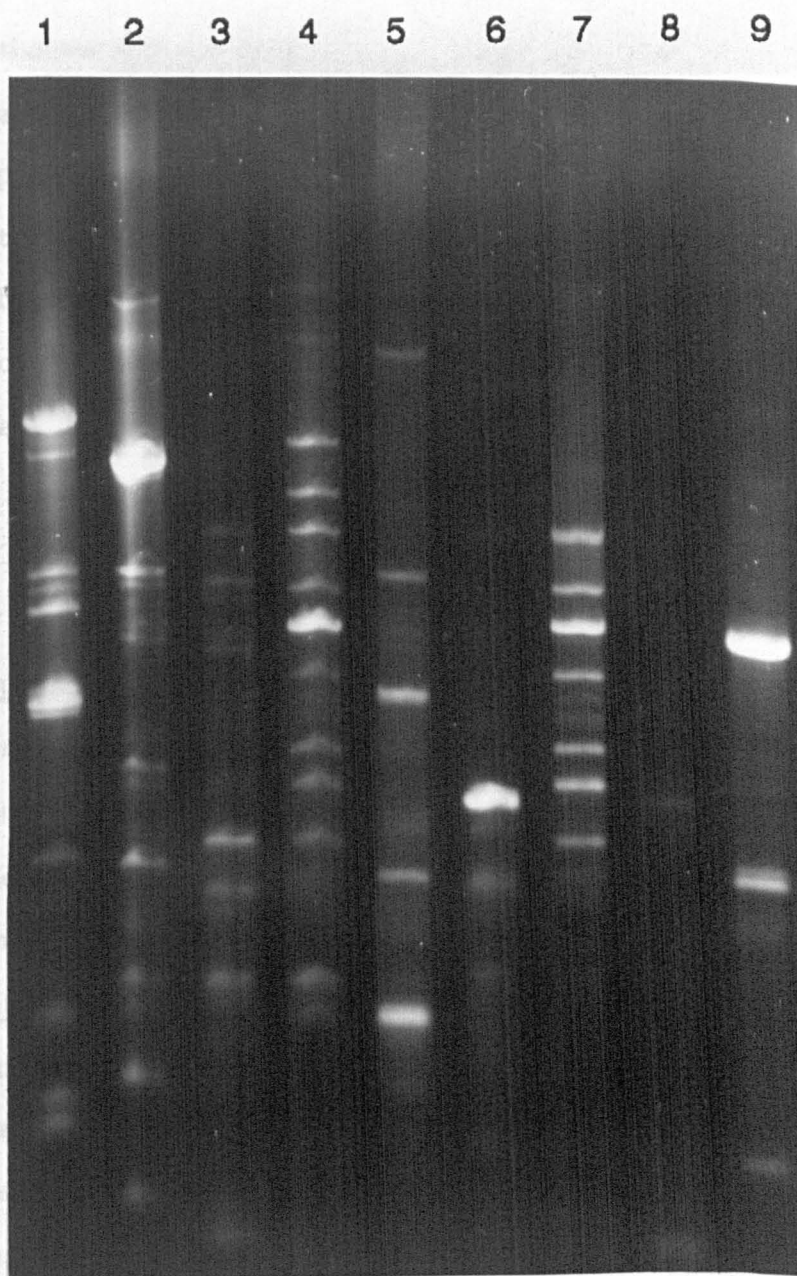
(control DNA)

Track 11: *M.trichosporium* OB3b DNA  
12: *M.extorquens* AM1 DNA  
13: *M.capsulatus* (Bath) DNA  
14: *E.coli* HB101 DNA

Unless otherwise stated, all amplification reactions were performed with 0.4  $\mu$ l each primer.







1: Erwinia  
2: DC2  
3: Klebsiella  
4: IR1  
5: Myxococcus

6: OB3b  
7: DR1  
8: MCB  
9: HB101

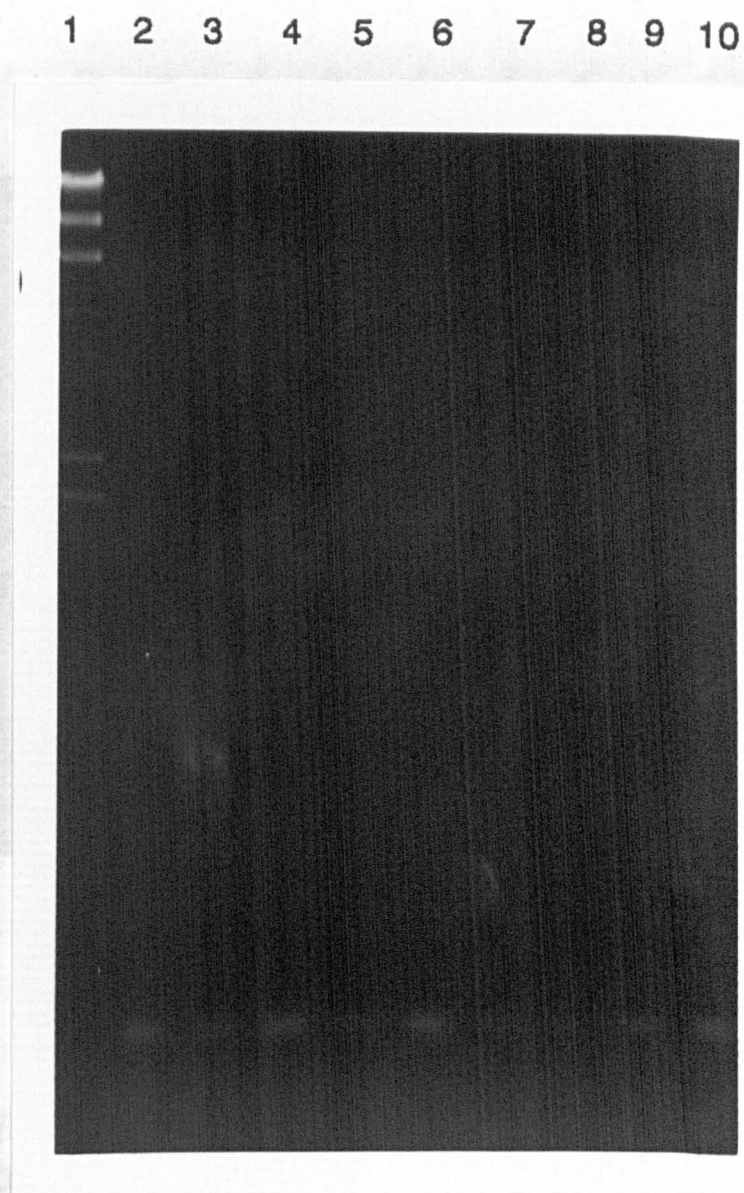
**Fig. 4.19 :** Non-methanotrophic DNA amplified with primers specific to *mmoY* (Markers not shown) For full names of bacteria, refer to fig. 4.11.



methanotrophic-specific DNA in sufficient quantity to be detected to enable hybridization with a mmoY-specific probe without the need for amplification, DNA isolated from environmental samples was digested with the restriction enzyme PstI and Southern blots prepared. Hybridization with the mmoY probe did not give a positive signal, showing that there was insufficient sMMO-specific DNA present in unamplified samples.

#### 4.3.5 Amplification of mmoZ - the $\gamma$ subunit

mmoZ is the gene that encodes the  $\gamma$  subunit. Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) show homology for mmoZ of 85% at the amino acid level. Two primers were chosen which would produce a PCR product of 348 bp. Initial results indicated that the stringency of annealing could be raised to 55°C. A product of the correct size was obtained after amplification of DNA isolated from Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b). This was also observed after amplification of environmental DNA samples (fig. 4.20). Hybridization with a mmoZ-specific probe, however, indicated that there was no homology with mmoZ. This result was confirmed after amplification of DNA from a range of non-methanotrophic bacteria (table 4.2) produced PCR products of a similar size (fig. 4.21). This indicated that the primers selected are amplifying a DNA sequence which is present in all the bacteria tested. As it is present in such a diverse group, from the enteric Escherichia coli and Erwinia caratovora, to Myxococcus xanthus, cyanobacteria and the Gram positive Streptomyces lividans, it is assumed that the amplified product is essential to a range of bacteria. Amplification produced a



1:  $\lambda$ HindIII markers

2: North Atlantic

3: Plymouth Sound

4: North Sea

5: River Leam

6: Chesford Grange pond

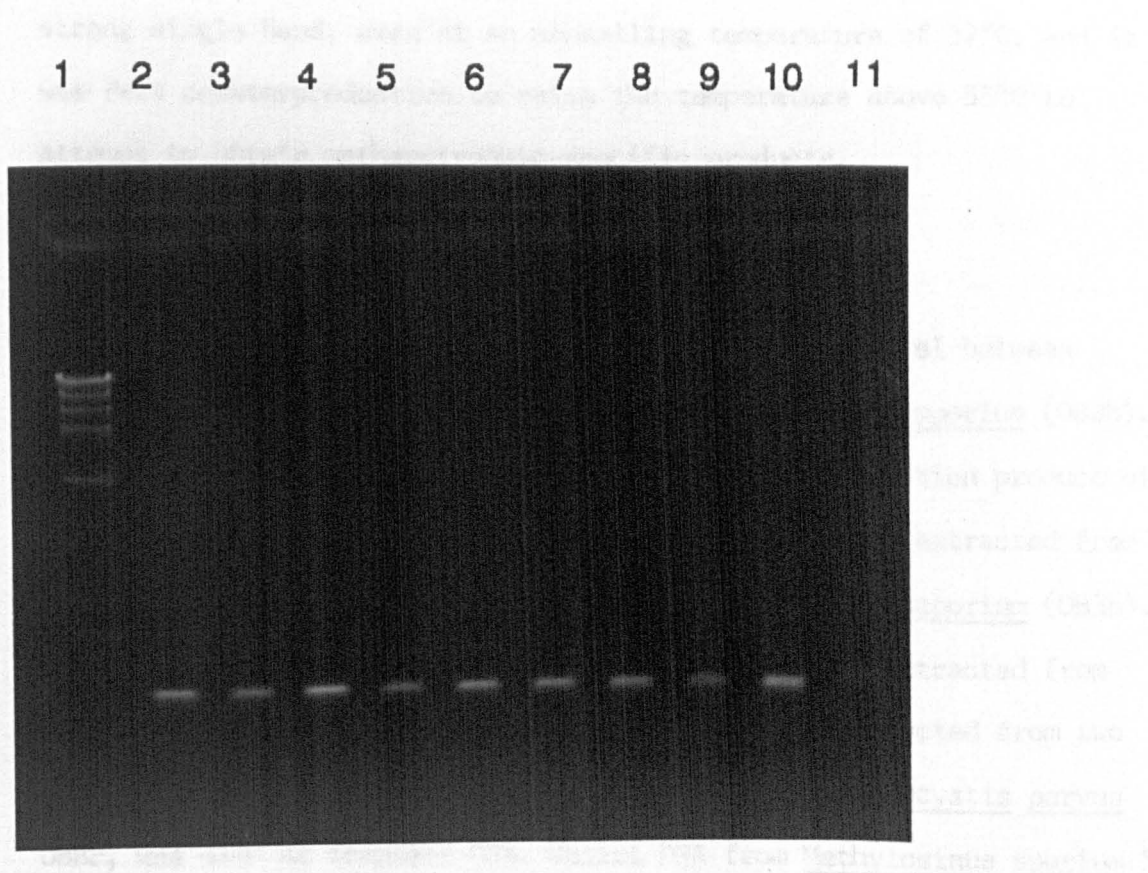
7: Newbold Comyn pond

8: East Site pond

9: Tocil Lake

10: MCB

**Fig. 4.20** Results of amplification of the gene encoding the  $\gamma$ -subunit (*mmoZ*)



- |                              |                        |
|------------------------------|------------------------|
| 1: $\lambda$ HindIII markers | 6: <i>Klebsiella</i>   |
| 2: DC2                       | 7: <i>Shigella</i>     |
| 3: HB101                     | 8: <i>Streptomyces</i> |
| 4: <i>Myxococcus</i>         | 9: <i>Erwinia</i>      |
| 5: AM1                       | 10: MCB                |
|                              | 11: OB3b               |

**Fig. 4.2/:** Results of amplification of the gene encoding the  $\delta$ -subunit (*mmoZ*)

For full names of bacteria, refer to fig. 4.11.

strong single band, even at an annealing temperature of 37°C, and it was felt counterproductive to raise the temperature above 55°C to attempt to obtain methanotrophic-specific products.

#### 4.3.6 Amplification of orfX - protein B of sMMO

orfX has a homology of 89.4% at the amino acid level between Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b). Two primers were selected which would give an amplification product of 290 bp. An amplification product was obtained with DNA extracted from Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b). However, no amplification could be obtained when DNA extracted from environmental samples was used as a template. DNA extracted from two other methanotrophs, Methylosinus sporium 5 and Methylocystis parvus OBBP, was used as template DNA. Whilst DNA from Methylosinus sporium 5 was amplified, DNA from Methylocystis parvus OBBP was not. As Methylocystis parvus OBBP does not contain sMMO (Stainthope et al, 1990b), the primers are specific to sMMO sequences. It is unclear as to why environmental samples could not be amplified with these primers.

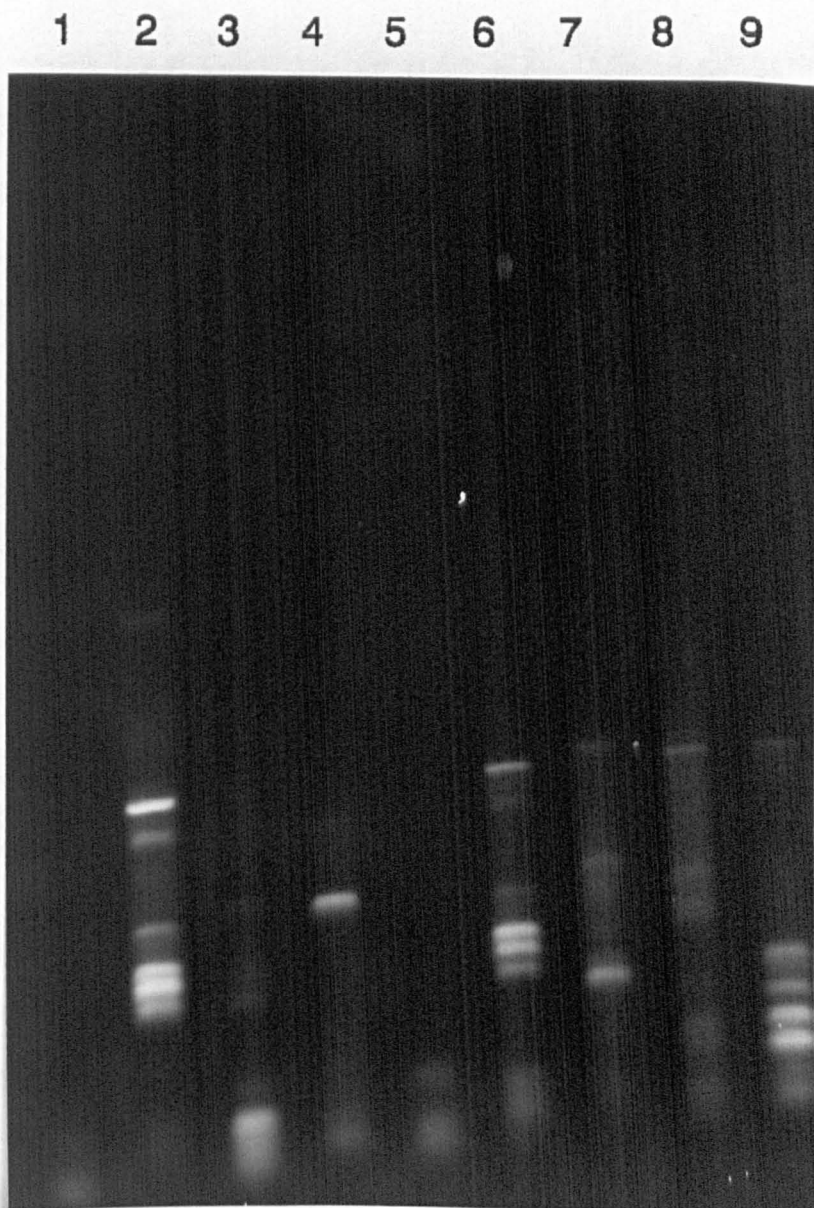
#### 4.3.7 Amplification of mmoC - protein C of sMMO

The homology between Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) for mmoC is 85% at the amino acid level. The primers chosen for amplification of mmoC would give a product of 562 bp. Amplification of DNA isolated from Methylococcus capsulatus (Bath) and Methylosinus trichosporium (OB3b) gave a product of approximately the right size, although there were non-specific

products also present (fig. 4.22). This was not improved by raising the annealing temperature to 55°C. Amplification of environmental DNA samples (see table 4.1) also gave a range of different bands. After hybridization with a mmoC specific probe, different sized bands appeared to hybridize (fig. 4.23). This indicates that the primers are amplifying non-methanotrophic DNA sequences in a similar way to mmoZ. In the case of mmoC, however, it is uncertain whether one or many genes are being amplified. mmoC has been shown to have homology with ferredoxins from plant, cyanobacteria and archaeobacterial origin (Stainthorpe et al, 1990).

#### 4.4 Conclusions from detection methodology

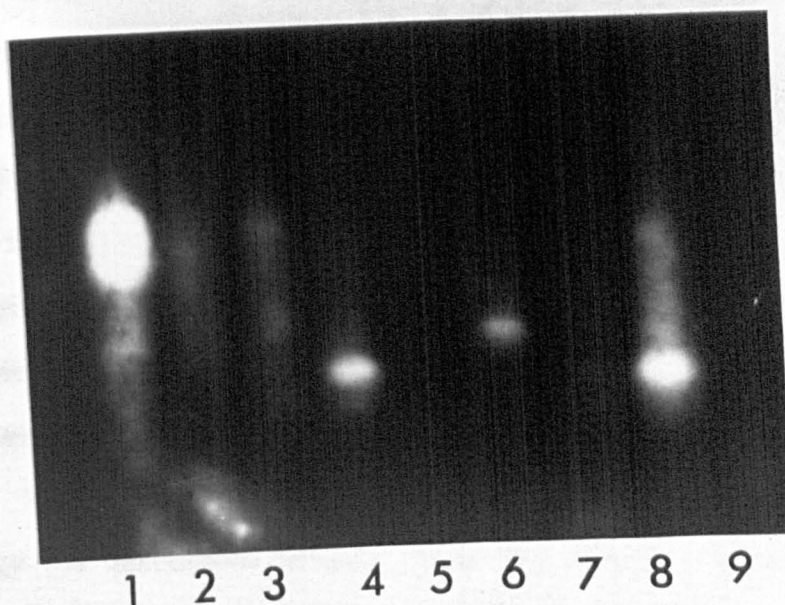
It has been shown that DNA isolated from environmental samples can be amplified by PCR. It has also been demonstrated that moxF can be used as a specific detection method for methylotrophic DNA, using DNA isolated from both purified bacterial cultures and from uncultivated environmental samples. An attempt has been made to enumerate methylotrophic bacteria in aquatic samples, with encouraging results. Further work is necessary to develop this methodology to its full potential. Several sets of primers have been investigated for their suitability for detection of bacteria containing sMMO. The gene which appears to offer a specific detection method for these bacteria is mmoY, encoding the subunit of protein A. This gene amplification system was specific for sMMO containing bacteria. Primers developed for amplification of mmoY and mmoC were found to amplify genes which were not unique to methanotrophs. Primers developed for amplification of mmoX and orfX were shown to amplify sMMO sequences, but further



- |                                  |   |
|----------------------------------|---|
| 1: DNA from North Atlantic       | 6: DNA from Newbold Comyn pond            |
| 2: DNA from Plymouth Sound       | 7: DNA from East Site pond                |
| 3: DNA from North Sea            | 8: DNA from Tocil Lake                    |
| 4: DNA from River Leam           | 9: <i>Methylosinus trichosporium</i> OB3b |
| 5: DNA from Chesford Grange pond |   |

**Fig. 4.22** : PCR-amplification of the gene encoding protein C (*mmoC*)  
 The gel was Southern blotted and probed with *mmoC*  
 Results of hybridization are shown in fig. 4.23.  
 (markers not shown)





- |                                  |   |
|----------------------------------|---|
| 1: DNA from North Atlantic       | 6: DNA from Newbold Comyn pond            |
| 2: DNA from Plymouth Sound       | 7: DNA from East Site pond                |
| 3: DNA from North Sea            | 8: DNA from Tocil Lake                    |
| 4: DNA from River Leam           | 9: <i>Methylosinus trichosporium</i> OB3b |
| 5: DNA from Chesford Grange pond |   |

**Fig. 4.23** Southern blot of agarose gel shown in fig. 4.22

The filter was washed at 45 C in 2 x SSC. At this low stringency, the radiolabelled bands are likely to be due to non-specific binding. The results shown here indicate that the primers used were not amplifying *mmoC*

work is necessary if these primers are to be developed for environmental detection. PCR has been used, therefore, as a method for detecting bacterial DNA which is specific for either MDH or sMMO in range of aquatic environments. Furthermore, the DNA can be quantified to give an estimate of population size. The work presented here has demonstrated the applications of PCR in the detection of methanotrophs and further development of the primers described here, or development of primers designed to amplify other methanotrophic-specific sequences, such as 16S rRNA sequences, will lead to a sensitive and accurate detection method.

There were two observations made whilst developing the methodology for detection, which, whilst not directly significant, were interesting. Firstly, when the 500 bp amplification products produced by methanol dehydrogenase-specific amplification were digested with the restriction enzyme HaeIII and the fragments run on an agarose gel, two patterns of bands were apparent. The freshwater bacterium Methylosinus trichosporium OB3b and DNA extracted from fresh water habitats produced a pattern of four bands, whilst the isolate IR1 and DNA isolated from marine habitats produced a pattern of two bands. Although methanol dehydrogenase appears to be highly conserved, and the amplification results presented here support that view, this may indicate that there are regions of divergence.

A second observation concerned the quality of the DNA extracted from environmental samples. For amplification by PCR, the template DNA needs to be relatively pure and uncontaminated by organic compounds. The method for extracting DNA used throughout the development of amplification techniques was that of Fuhrman et al (1988), which



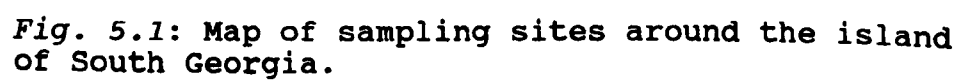
involves harvesting by filtration. This method was not designed specifically for amplification methods. A second method (Atlas & Bej, 1990) was compared with this. This second method involved harvesting by centrifugation and was designed to provide DNA suitable for amplification. However, whilst the DNA yield from both methods was similar, DNA purified by the centrifugation method could not be amplified with moxF-specific primers.

THE OCCURRENCE OF METHANOTROPHS  
IN THE SOUTHERN OCEAN

### 5.1 Environmental sampling in the Southern Ocean

The distribution and activity of methanotrophs in the Southern Ocean was investigated between February and March 1990 during a British Antarctic Survey cruise (OBP10). Two areas were sampled; the South Georgia north coast and the Weddell Sea (off the Antarctic peninsula) and sampling sites are shown in figs. 5.1 and 5.2. Sampling was carried out during the mid- to late-southern summer. The Southern Ocean was investigated because it is a region of very clean (i.e. unpolluted) water, a factor of some importance when investigating hydrocarbon utilization. It was felt that a survey of methanotrophic activity would indicate the importance of the role of methanotrophs in this environment.

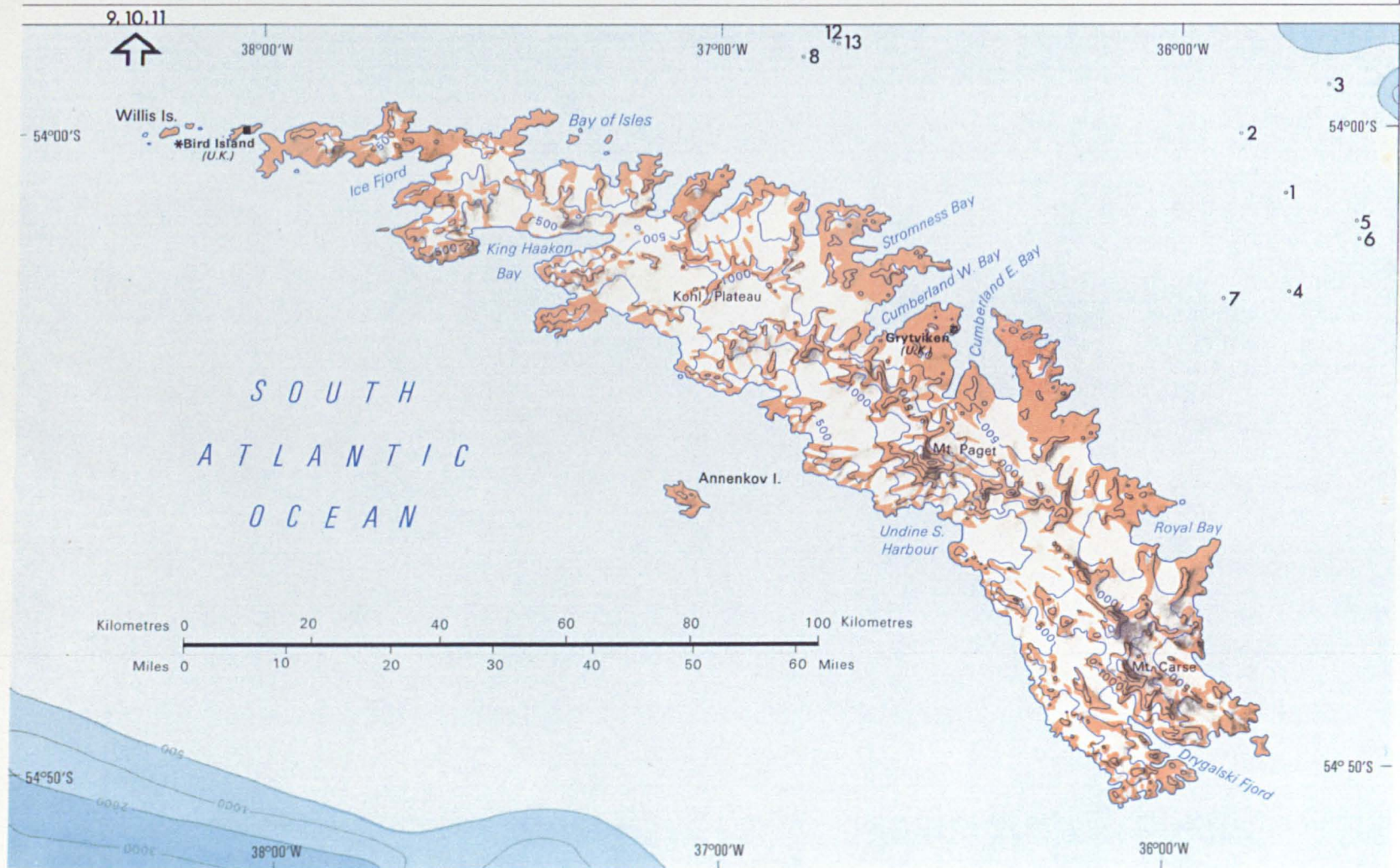
The survey investigated three main parameters. Total heterotrophic bacterial activity was estimated by following the uptake of tritiated thymidine ( $^3\text{TdR}$ ). Although this method may underestimate the true activity (as discussed in section 1.7.3), its simplicity ensures that this method still remains the primary means of measuring heterotrophic activity. At some of the sites, bacterial activity measurements were compared to direct bacterial counts, obtained by staining fixed water samples with acridine orange and counting the stained bacteria. Secondly, the methane oxidation rate of a volume of water was determined. This was carried out by following the uptake of radiolabelled methane ( $^{14}\text{CH}_4$ ) and its conversion to cell carbon and  $\text{CO}_2$ . This method of estimating methane oxidation rates is also a common method (for example, see Ward et al, 1987). Finally, the methane concentration of the water was determined by a head-space equilibrium technique.



**Fig. 5.1: Map of sampling sites around the island of South Georgia.**

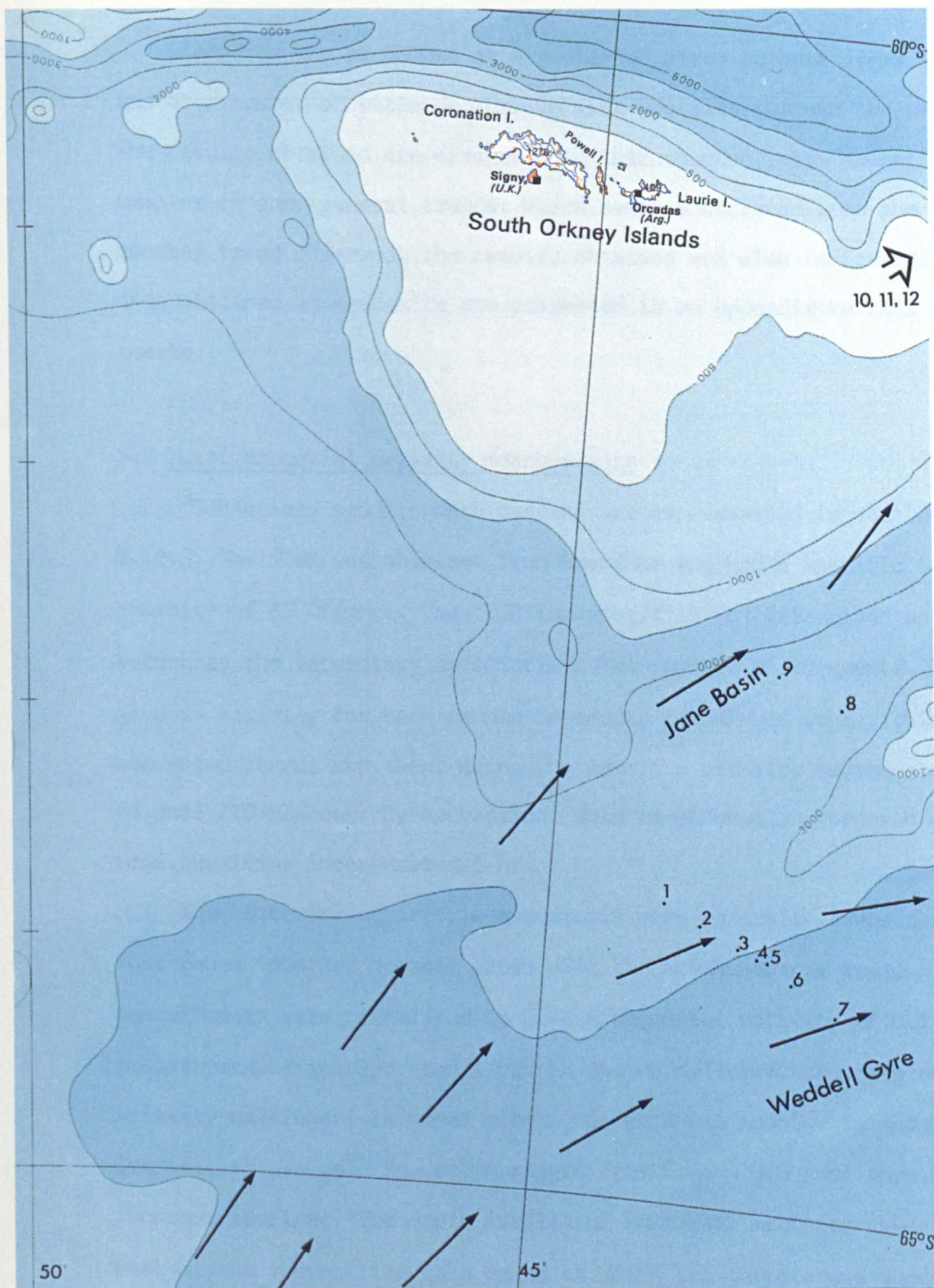
# SOUTH GEORGIA

Scale 1:1,000,000



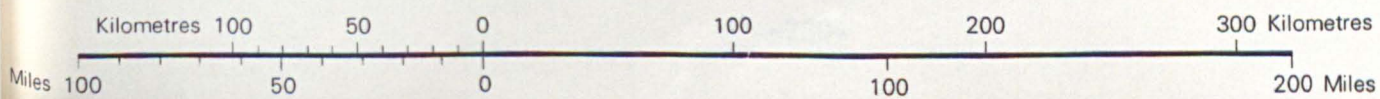
***Fig: 5.2: Map of sampling sites in the Weddell Sea, off the Antarctic peninsular.***





Polar Stereographic Projection

Scale 1:3,000,000



Sampling was performed at a number of sites in both areas tested and at a number of water depths, to give profiles through the water. The results obtained are discussed in this chapter, with specific samples to show general trends. Where samples differed from the general trend observed, the results obtained are also indicated. The data obtained at each site are presented in an appendix to this thesis.

## 5.2 Total bacterial activity measurements

$^3\text{TdR}$ -uptake studies were carried out as described in section 2.12.3. The  $^3\text{TdR}$  was obtained from Amersham and had a specific activity of 85 Ci/mmol. The  $^3\text{TdR}$  incorporation was determined on return to the laboratory by scintillation counter measurements. This gave an activity for each sample in counts per minute (cpm). This was converted to uCi and then, using the specific activity quoted above, to nmol./10 ml/hour. By convention, this is generally expressed as pmol.thymidine incorporated/l/hr.

The bacterial activity measurements were generally lower than that found in other oceanic areas. For the South Georgia area, measurements were calculated to give a bacterial activity of 0.1 - 1.0 pmol.thymidine incorporated/l/hr. In the Weddell Sea, the rate of activity was lower. In some sites, the rate was too low to measure. Overall, the Weddell Sea rates ranged from 0.04 - 0.6 pmol.thymidine incorporated/l/hr. The depth profile of bacterial activity revealed a peak at the thermocline (the depth at which the temperature suddenly drops, above which the water is warmed by solar heating). There was a second rise in bacterial activity associated with the sediment layers.



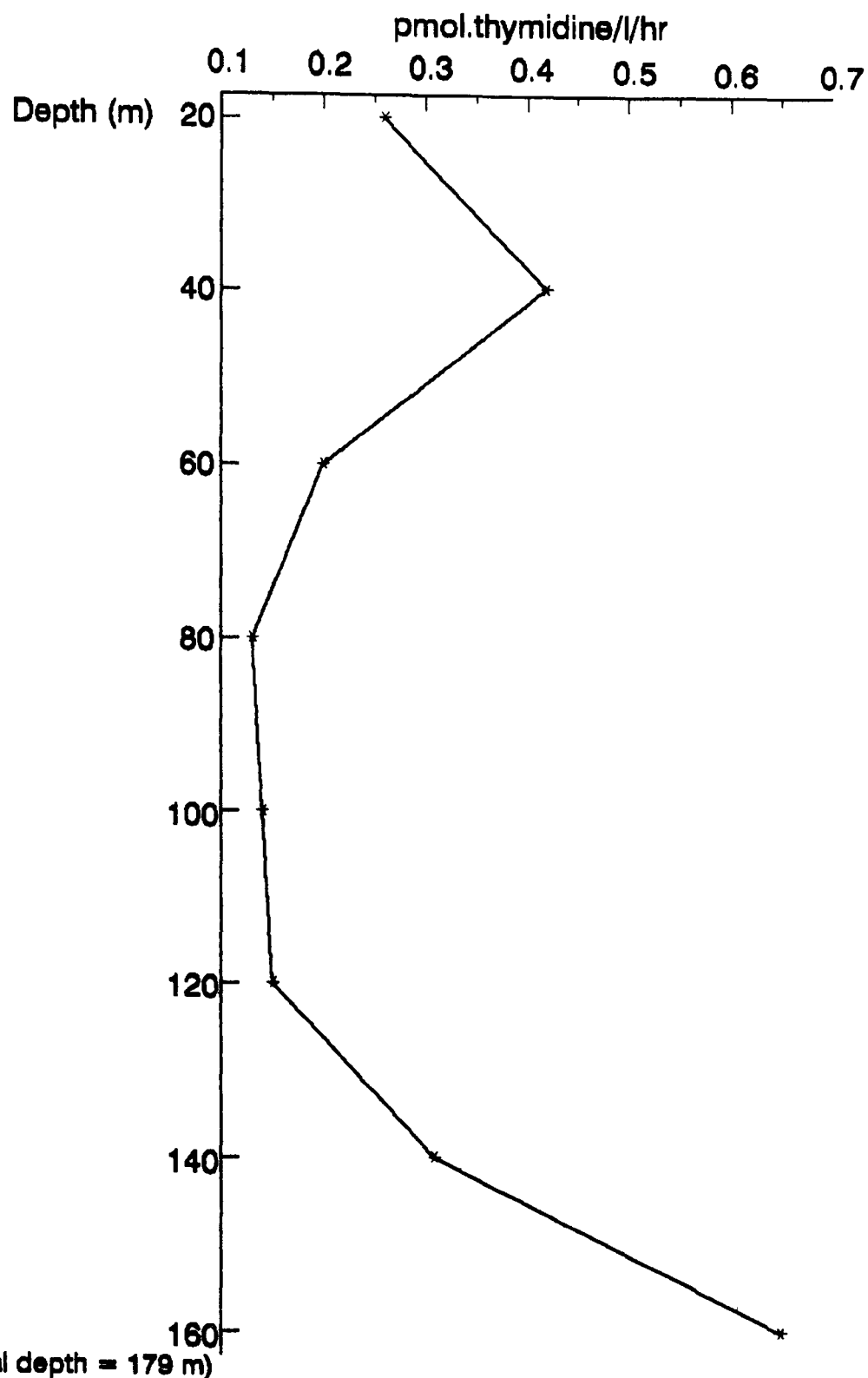
This was only seen occasionally, as it was difficult to sample so close to the bottom. The distribution of bacterial activity in the South Georgia area is shown in fig. 5.3. This is a clear illustration of the bacterial activity/depth profile in this area. Fig. 5.4 illustrates the bacterial activity in the Weddell Sea and shows the lower rates observed in this area. Bacterial activity rates in other oceanic areas are reportedly higher than the rates shown here. Values for the Celtic Sea were found to be up to 5 pmol.thymidine incorporated/l/hr (Joint & Pomroy, 1987). Bietri Bay, Ivory Coast, showed values of up to 2140 pmol.thymidine incorporated/l/hr and coastal sea water from the same area had a value of 17.6 pmol.incorporated/l/hr (Torreton & Bouvy, 1991). Values of <80 pmol.thymidine incorporated/l/hr were reported for Chesapeake Bay by Chin-Leo & Kirchman (1988), although this averaged around 30 pmol.thymidine incorporated/l/hr. However, although the values for the Southern Ocean samples are low, they are in good agreement with values obtained from the Southern Ocean in 1988 (J.Priddle, pers. comm.), where samples ranged from 0.06 - 3.5 pmol.thymidine incorporated/l/hr.

Direct bacterial counts were performed on several of the samples. A representative depth profile is illustrated in fig. 5.5. The direct bacterial count results follow the pattern seen by  $^3\text{TdR}$ -uptake, in that when high values of  $^3\text{TdR}$  incorporation were measured, the direct bacterial counts were also high.

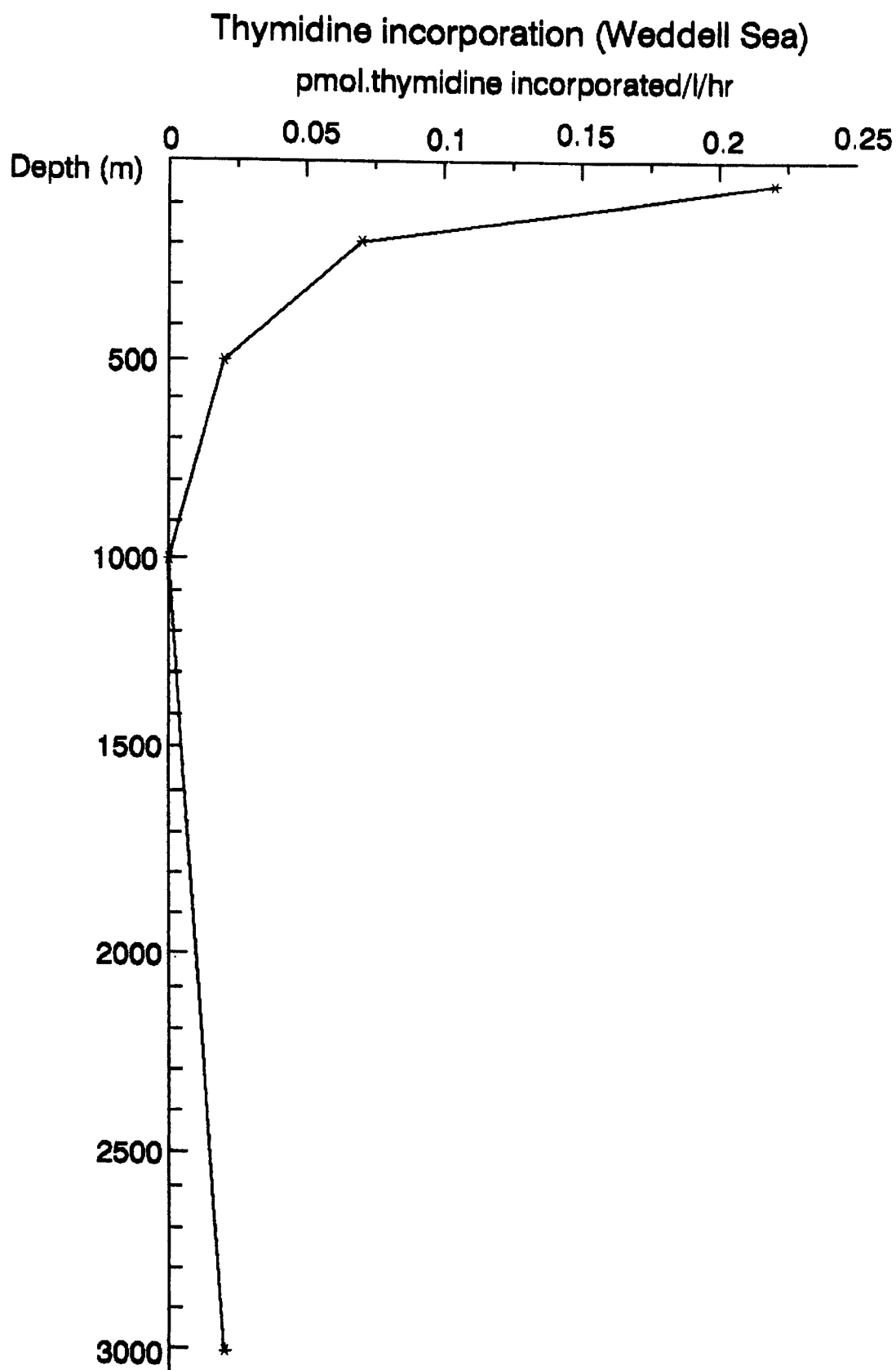
### 5.3 Methane oxidation rates

The  $^{14}\text{CH}_4$  was obtained from Amersham and had a specific activity of 55.9 mCi/mmol. The  $^{14}\text{CH}_4$ -uptake was calculated by scintillation

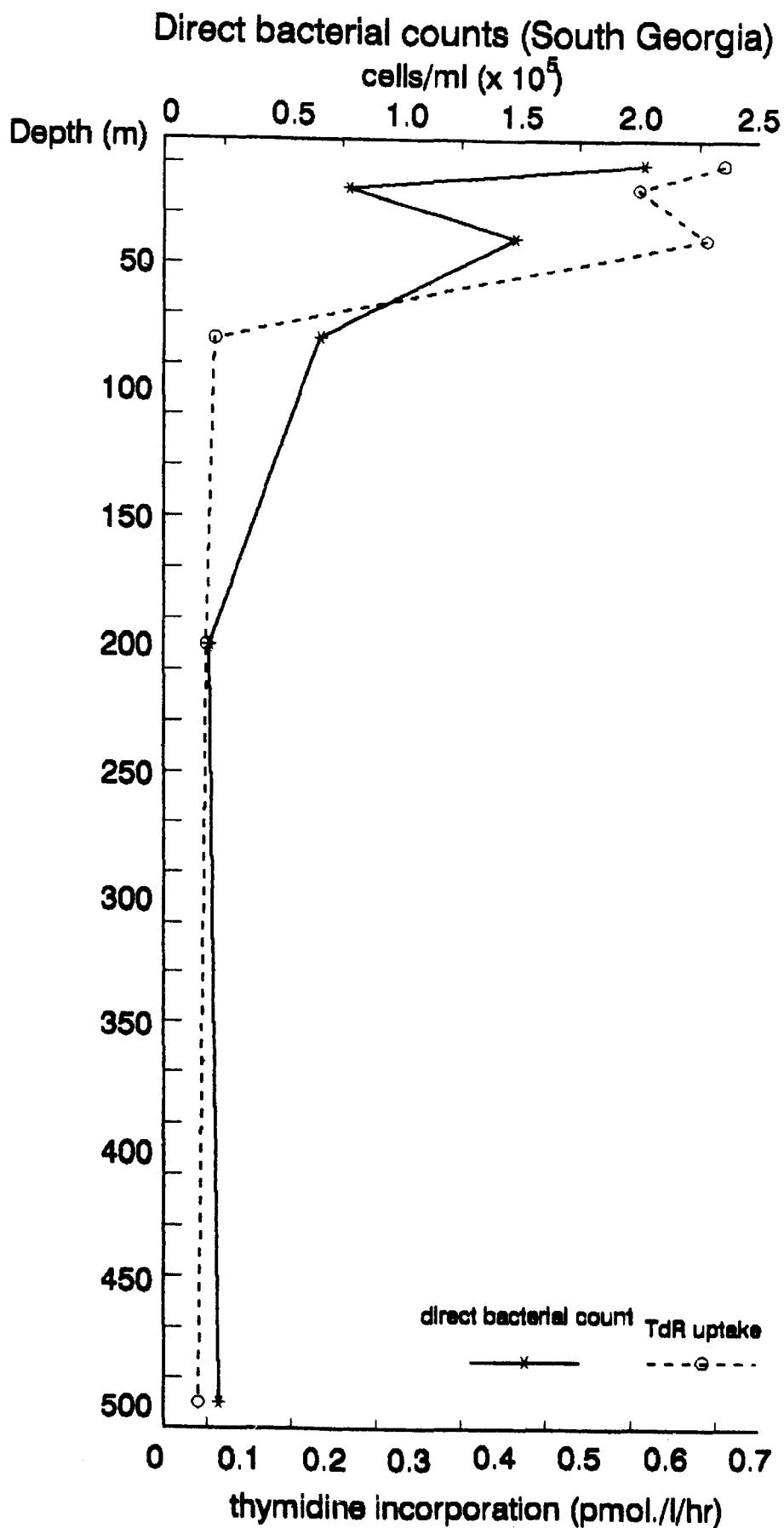
# Thymidine incorporation (South Georgia)



**Fig. 5.3 :** Thymidine incorporation at site 7, South Georgia. Activity peaks at the thermocline and again at the bottom of the water column.



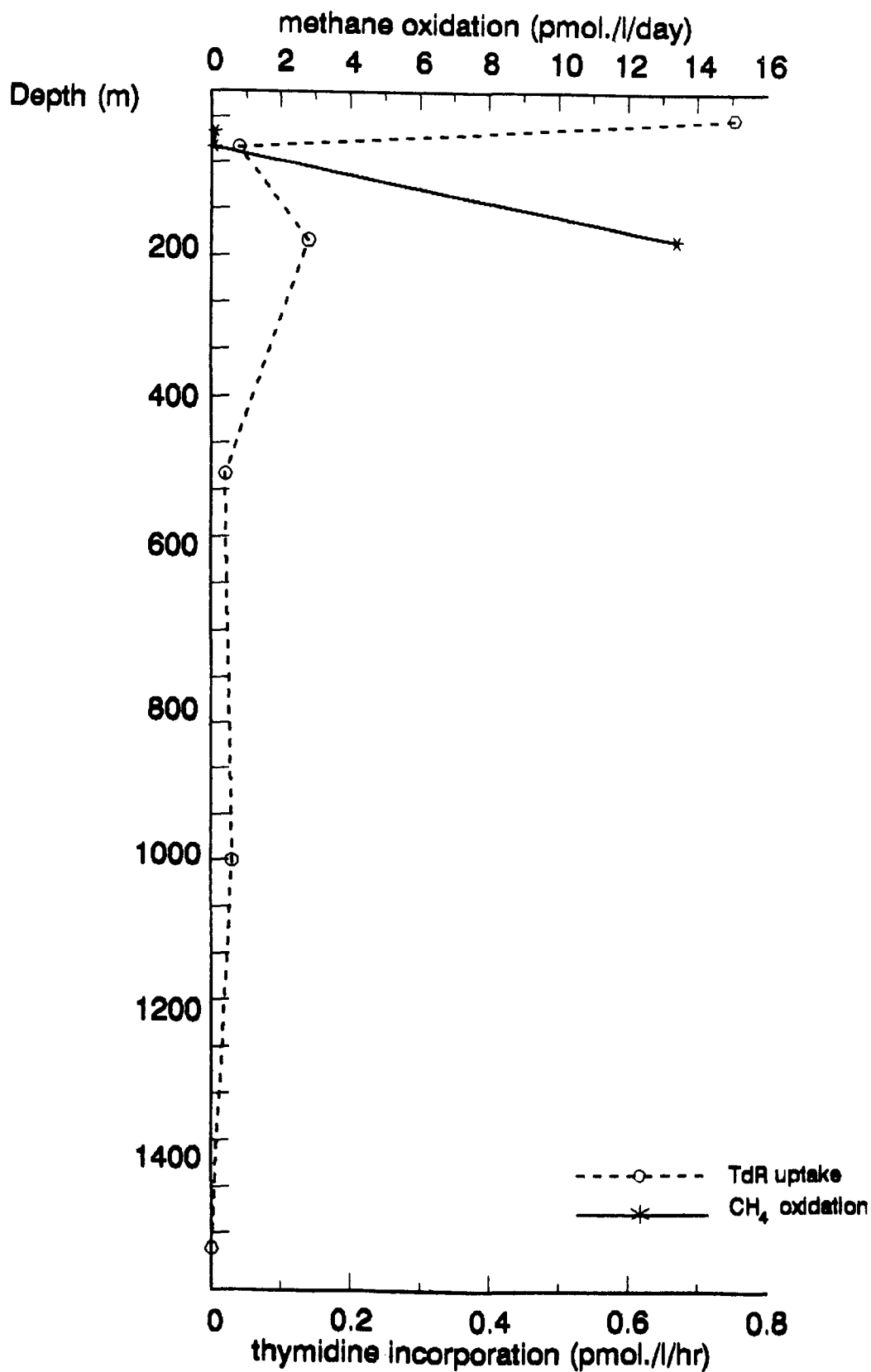
**Fig. 5.4:** Thymidine incorporation at site 24, Weddell Sea.



**Fig. 5.5 : Direct bacterial counts from site 11, South Georgia.**

counting measurements on return to the laboratory. In a similar manner to that described for  $^3\text{TdR}$ -uptake, the rate of methane oxidation activity was determined as pmol.methane utilized/l/day. The values obtained from these measurements were generally low and ranged from 0.05 - 0.7 pmol.methane utilized/l/day in the South Georgia area. In the Weddell sea, the values ranged between 0.05 - 0.76 pmol.methane utilized/l/day, and therefore, in contrast to the thymidine uptake, appears similar. In two of the South Georgia sites, the methane oxidation rates were much higher. At a site to the North west of the island, a rate of 13.4 pmol.methane utilized/l/day was recorded at a depth of 200 m in a total depth of 1595 m. The bacterial activity determined by  $^3\text{TdR}$  incorporation was also elevated, but methane concentration did not appear to be higher than expected. This is illustrated in figure 5.6. The second site with a higher activity was directly north from the island. This site had a methane oxidation rate of 4.1 pmol.methane utilized/l/day. The sample was from a depth of 220 m in a total depth of 235 m and was not associated with elevated  $^3\text{TdR}$ -uptake, although the methane concentration appeared to be much higher for this sample. One of the Weddell Sea samples also appeared to have a slightly higher methane oxidation rate of 2.8 pmol.methane utilized/l/day, but this was not associated with high levels for either methane concentration or  $^3\text{TdR}$  incorporation. Methane oxidation rates at different depths showed no clear pattern, although the rate was slightly elevated in samples from the bottom of the water column. There are few reported methane oxidation rate values for oceanic waters and there are several different methods for determining oxidation rates.

# Methane oxidation rate and thymidine uptake (South Georgia)



**Fig. 5.6** Methane oxidation rates from site 10, South Georgia.

#### 5.4 Methane content of seawater samples

The methane content of seawater samples was measured as described in section 2.12.2, using a head-space equilibrium technique. This technique had been used previously by Owens et al (1991) to determine the methane concentration in the Indian Ocean and the Arabian Sea. The measurement of methane in the Southern Ocean by this technique, however, did not seem to give believable results. The cause of this is uncertain; the gas chromatograph used may have not functioned correctly, or the concentration of methane in the Southern Ocean may have been too low to measure using this technique. Both areas previously sampled in this way were found to have a high methane concentration. The figures are still subject to analysis and if, after further study, the data are found to be publishable, it will be submitted to a suitable journal. The raw data from this survey are included in the appendix. In the absence of reliable methane concentration data, general observations about the pattern of methane distribution may still be made. Firstly, all areas seemed to have similar concentrations of methane. Secondly, methane concentration increased with depth in the shallower waters of South Georgia, a phenomenon that was not apparent in the Weddell Sea. The high methane oxidation rate observed in site 12 was associated with a much higher methane concentration. There were no other sites of raised or lowered methane concentration.

#### 5.5 Conclusions to environmental sampling

The methane oxidation data showed that there were pockets of high rates of methanotrophic activity. Although one site was linked with

increased heterotrophic activity (site 10), the other two high methane oxidation sites (site 12, South Georgia and site 16, Weddell Sea) were not. Site 12, South Georgia, may have been linked to increased methane concentration, but no firm conclusions can be drawn from this. It would be interesting to conduct a similar survey to compare the methane oxidation rates measured by  $^{14}\text{CH}_4$  uptake with data from the enumeration techniques developed. For any firm conclusions to be drawn from such data, however, methane concentration methodology needs to be improved.



## DISCUSSION

### 6.1 Discussion of results and implications for further research

Recently, the role of methane in global warming has been of increasing importance. The concentration of methane in the environment is controlled by production and oxidation by both chemical and biological processes. Methane oxidation by bacteria is an important part of the global methane cycling and has been well studied in terrestrial and fresh water environments by a number of workers. The role of methanotrophs in the marine environment, however, is still to be determined. The work described here is an attempt to investigate the occurrence of methane-oxidizing bacteria in the marine environment and to examine their characteristics.

### 6.2 Enrichment and isolation of marine methanotrophs

Enrichment for methanotrophs in liquid cultures was relatively successful for samples from the marine environment. However, isolation of pure cultures from enrichment cultures was hampered because the methanotrophs did not grow well on solid media. Traditional isolation methods have relied on cultivation on solid media. This showed that methanotrophs were present in a sample and also gave an indication of their abundance in that sample. The observation that methanotrophs isolated did not grow on a range of solid media, in the marine environment at least, indicates that traditional cultivation of methanotrophs fails to isolate many methanotrophic strains present in the environment.

Results presented in this study show that methanotrophs can be isolated from marine environments by liquid enrichment techniques. Isolation of pure methanotrophic cultures were obtained by use of

semi-solid media. It is likely that, although liquid enrichment techniques allow isolation of representative methanotrophs, there are methanotroph strains that can not be isolated in this way. It was therefore necessary to develop detection techniques which do not rely on cultivation of the bacteria. The methods developed are discussed below.

### 6.3 Characterization of marine methanotrophs

The characterization of marine methanotrophs has been discussed in detail in chapter 4 and is only summarized in this section. The bacteria isolated from Plymouth Sound were found to be typical of Type I methanotrophs by examination of the carbon assimilation pathways and by mol% G+C ratio. There were several differences to previously described methanotrophs from fresh water environments. Firstly, they had an absolute requirement for sodium chloride. The range of salt concentrations tolerated by both isolates was that expected of true marine bacteria. Secondly, both organisms had unusual nitrogen assimilation pathways. Unlike most bacteria, these isolate appeared to assimilate ammonia in the cell by glutamate dehydrogenase and not by the GS/GOGAT pathway. It was also observed that both bacteria could not utilize ammonia as a sole nitrogen source, although there was no evidence that ammonia was toxic to the cells.

### 6.4 Detection of marine methylotrophs

It has been shown in this study that both methylotrophs and, more specifically, methanotrophs can be detected by utilizing molecular techniques. DNA isolated from pure cultures of methylotrophs and

methanotrophs and also from environmental samples can be amplified by PCR using primers which are specifically homologous to genes encoding structural proteins of methanol dehydrogenase and soluble methane monooxygenase. The primers have been used to synthesise DNA products which were shown to hybridize to specific probes for the genes. When DNA isolated from bacteria which are not known to contain methanol dehydrogenase or methane monooxygenase was amplified by the specific primers, a specific product was not observed. This illustrates that the methods described in this study are suitable for specific detection of methylotrophs or methanotrophs. It was also demonstrated that these techniques could be adapted to quantify methylotrophs or methanotrophs present in a sample.

#### 6.5 Occurrence of methanotrophs in the Southern Ocean

Methanotrophic activity was measured in all sites of the Southern Ocean sampled. Increased levels of methanotrophic activity was observed in several areas but was not linked strongly with a single parameter. Other conclusions from this study are made in section 5.5 and will not be discussed any further here.

#### 6.6 Further work

This study has raised a number of questions which need to be addressed in future experiments. The characterization of the bacteria isolated shows that these methanotrophs have unusual pathways of nitrogen metabolism. It still remains to be seen if this is a widespread phenomenon in marine bacteria, or whether it is unique to

marine methanotrophs, or to these isolates. Nitrogen metabolism in these bacteria may be more complicated than this study has shown and this needs to be examined if a full picture of marine methanotroph activity is to be examined.

The detection of methylo-trophs by amplification of methanol dehydrogenase-specific sequences has been shown to be a suitable method for both detection and enumeration of methylo-trophs in aquatic environments. It would be interesting to continue the detection of methylo-trophs in habitats not yet examined, for example, hydrothermal vents and hot springs. This would allow us to build a picture of the occurrence and numbers of methylo-trophs in different environments. The amplification of sMMO-specific sequences, however, only offers us a limited detection of methanotrophs which contain soluble methane monooxygenase. At present, data from characterized methanotrophs indicate that sMMO is present in only a small proportion of methanotrophs. As the particulate form of MMO has not yet been isolated, it seems unlikely that primers specific to the genes encoding pMMO will be developed in the near future. There are, however, alternatives to amplifying pMMO gene sequences for detection. Recent publications describe the sequence data obtained from 16S rRNA isolated from several methanotrophic species. These sequences contain regions specific to methanotrophs. Primers homologous to these regions will allow detection of all methanotrophs which contain the sequences. It would then be possible to compare the number of bacteria in an environment amplified with the 16S rRNA-specific primers with that amplified by the sMMO-specific primers to gain an insight into the proportion of methanotrophs which possess the soluble form of methane

monooxygenase. In addition, the proportion may vary with habitat and this could also be investigated.

Finally, by investigating the occurrence of methanotrophic activity in a range of marine environments by traditional  $^{14}\text{C}$ -methane uptake and by the molecular detection techniques developed in this study, the activity of methanotrophic bacteria can be determined. Together with methane concentration data and bacterial activity measurements, this will lead to an understanding of the role of methanotrophs in the cycling of methane in the marine environment and their abundance in bacterioplankton.

## APPENDIX

The results presented in this appendix are the measurements taken from sites in the Southern Ocean during a two month cruise on the British Antarctic survey research ship RRS John Biscoe (cruise OBP10). The areas sampled are shown in figs. 5.1 and 5.2. The results are calculated as described in chapter 5, together with a discussion of the main conclusions from this work.

SOUTH GEORGIA SAMPLES

EVENT #1 050290 54.06 lat: 35.46 long

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
5		0
20		0.48
40		0.26
60		1.00
100		0.43

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
5	0.14
20	0.21
40	0.19
60	0.19
100	0.20

CH <sub>4</sub> oxidation rates		
DEPTH(m)	pmol	l <sup>-1</sup> day <sup>-1</sup>
5		0.067
20		0.051
40		0.047
60		0.041
100		0.047

Direct Counts	
DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
5	1.98
20	1.78
60	1.62
100	1.25

TOTAL DEPTH = 225m

EVENT #2 070290 53.59 lat: 35.51 long

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
20		0.40
50		0.31
100		0.12

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
20	0.88
50	0.11
100	0.13

CH <sub>4</sub> oxidation rates		
DEPTH(m)	pmol	l <sup>-1</sup> day <sup>-1</sup>
20		L O S T
50		0.35
100		0.17

TOTAL DEPTH = 244m

EVENT #3 070290 53.57 lat: 35.39 long

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
20		0.21
50		0.44
100		0.16

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
20	0.06
50	0.08
100	0.12



CH <sub>4</sub> oxidation rates		
DEPTH(m)	pmol	l <sup>-1</sup> day <sup>-1</sup>
20		0.69
50		0.25
100		0.31

TOTAL DEPTH = 224m

---

EVENT #4      080290      54.13 lat: 35.44 long

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
20		0
60		0.11
120		0.14
140		0.13
180		0.84
225		0.67

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
5	0.472
20	0.383
40	0.469
60	0.397
80	0.395
100	0.355
120	0.356
140	0.327
160	0.338
180	0.321
200	0.421
225	0.395

CH <sub>4</sub> oxidation rates		
DEPTH(m)	pmol	l <sup>-1</sup> day <sup>-1</sup>
225		0.26

Direct Counts	
DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
20	1.54
60	0.99
160	0.67

TOTAL DEPTH = 232m

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EVENT #5      100290      54.079 lat: 35.39 long

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
5		0.24
20		0.45
30		0.03
60		0.29
100		0.17
140		0.12

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
5	0.14
10	0.075
20	0.092
30	0.094
40	0.090
50	0.12
60	0.12
80	0.08
100	0.10
120	0.11
140	0.10
150	0.13

CH <sub>4</sub> oxidation rates		
DEPTH(m)	pmol	l <sup>-1</sup> day <sup>-1</sup>
30		0.06
60		0.35
100		0.21

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EVENT #6      110290      54.09 lat: 35.35 long

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
20		0.26
40		0.42
60		0.20
80		0.13
10		0.14
120		0.15
140		0.31
160		0.65

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
40	0.1603
40	0.160
60	0.146
80	0.176
100	0.079

CH <sub>4</sub> oxidation rates		
DEPTH(m)	pmol	l <sup>-1</sup> day <sup>-1</sup>
60		0.05
120		0.07
140		0.08

Direct Counts	
DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
20	0.37
40	0.31
60	0.66
80	0.98
120	1.02
160	1.18

TOTAL DEPTH = 179m

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EVENT #7      120290      54.14 lat: 35.53 long

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
10		0.50
40		0.53
100		0.26
160		0.17

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
10	0.12
20	0.12
40	0.124
60	0.14
100	0.16
160	0.18

TOTAL DEPTH = 227m

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EVENT #8      130290      53.55 lat: 36.48 long

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol	l <sup>-1</sup>
10		0.78	10		0.163
40		0.28	20		0.18
100		0.07	40		0.22
160		0.07	60		0.12
			100		0.09
			160		0.164

TOTAL DEPTH = 204m

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EVENT #9      130290      53.49 lat: 38.17 long

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol	l <sup>-1</sup>
10		0.74	10		0.12
40		0.65	20		0.05
100		0.12	40		0.29
160		0.12	60		0.10
			100		0.14
			160		0.23

TOTAL DEPTH = 194m

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EVENT #10      150290      53.40 lat: 38.34 long

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol	l <sup>-1</sup>
10		0.67	10		0.11
20		0.56	20		0.11
40		0.64	40		0.10
80		0.06	80		0.15
200		0.05	200		0.15
500		0.04	500		0.21

Direct Counts

DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
10	2.02
20	0.78
40	1.48
80	0.66
200	0.19
500	0.23

TOTAL DEPTH = 1516m

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EVENT #11 160290 53.39 lat: 38.34 long

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
40		0.75	5	0.14
80		0.04	10	0.13
200		0.14	20	0.25
500		0.02	30	0.13
1000		0.03	40	0.16
1500		0	60	0.10
			80	0.14
			100	0.13
			200	0.12
			500	0.12
			1000	0.18
			1500	0.22

CH <sub>4</sub> oxidation rates	
DEPTH(m)	pmol l <sup>-1</sup> day <sup>-1</sup>
60	0.11
80	0.06
200	13.40

TOTAL DEPTH = 1595m

EVENT #12 190290 53.53 lat: 36.47 long

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
40		0.39	20	0.10
120		0.07	40	0.09
220		0.05	120	0.24
			150	0.12
			220	0.24

CH <sub>4</sub> oxidation rates		Direct Counts	
DEPTH(m)	pmol l <sup>-1</sup> day <sup>-1</sup> (m)	DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
40	0.16	40	1.17
120	0.12	120	0.65
220	4.05	220	0.54

EVENT #13 210290 (water sampled in area of Krill swarm)

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
20		0.61	20	0.10
40		0.79	40	0.12
60		0.61	60	0.14
100		0.14	100	0.13
240		0.13	240	0.05
20		0.86	20	0.09
60		0.23	60	0.10
155		0.11	155	0.16

CH<sub>4</sub> oxidation rates

DEPTH(m)	pmol l <sup>-1</sup> day <sup>-1</sup>
20	0.14
100	0.11
240	0.55

WEDDELL SEA SAMPLES

EVENT #1 040390 60.11 lat: 43.34 long

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
100	0.28		20	0.08
1000	0.05		100	0.11
2500	0		1000	0.09
4000	0		2500	0.08
5000	0.07		4000	0.08
			5000	0.13

CH <sub>4</sub> oxidation rates	
DEPTH(m)	pmol l <sup>-1</sup> day <sup>-1</sup>
1000	0.13
5000	0.06

EVENT #2 040390

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
200	0.21		200	0.14
1500	0.04		1500	0.04
3000	0		3000	0.07
5085	0.11		4669	0.06
			4000	0.08
			5085	0.12

CH <sub>4</sub> oxidation rates	
DEPTH(m)	pmol l <sup>-1</sup> day <sup>-1</sup>
200	0.07
5085	0.33

EVENT #3 050390 63.02 lat: 43.07 long

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
100	0.25		100	0.2
500	0		500	0.06
2000	0		1200	0.05
3960	0.19		2000	0.08
			2893	0.16
			3960	0.06

CH <sub>4</sub> oxidation rates	
DEPTH(m)	pmol l <sup>-1</sup> day <sup>-1</sup>
500	2.77
200	0.82

Direct Counts	
DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
100	0.21

EVENT #4 060390

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
100		0	100	0.09
1000		0	1000	0.10
3745		0	2000	0.076
			2955	0.09
			3745	0.09

EVENT #5 060390

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
100		0	100	0.1
200		0	200	0.07
500		0	500	0.09
1000		0	1000	0.06
2000		0	2000	0.07
			2867	0.07
			3252	0.10
			3861	0.10

CH <sub>4</sub> oxidation rates	
DEPTH(m)	pmol l <sup>-1</sup> day <sup>-1</sup>
100	0.34
500	0.047
2000	L O S T

EVENT #6 070390

<sup>3</sup> TdR accumulation			CH <sub>4</sub> concentration	
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>	DEPTH(m)	nmol l <sup>-1</sup>
20		0.46	20	0.12
100		0.11	100	0.12
1000		0	1000	0.07
3000		0	3000	0.09
4821		0.06	4821	0.07

Direct Counts	
DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
20	0.76
100	0.28

EVENT #7 080390

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
20		0.61
100		0.42
1000		0
2000		0.06
3000		0

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
20	0.16
100	0.10
1000	0.09
2000	0.09
3000	0.05
3820	0.07
4605	0.06

Direct Counts

DEPTH(m)	CELLS/ml X 10 <sup>5</sup>
20	2.25
100	0.38

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EVENT #8 080390

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
50		0.35
500		0.04
1000		0
2000		0
3000		0.21

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
50	0.09
500	0.08
1000	0.08
2000	0.07
3000	0.08
3632	0.06
4612	0.12

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EVENT #9 090390

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
50		0.34
500		0
1000		0.02
3319		0

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
50	0.09
500	0.07
1000	0.061
2000	0.09
2538	0.11
3319	0.09

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EVENT #10 110390 [may have hit bottom with deepest bottle]

<sup>3</sup> TdR accumulation		
DEPTH(m)	nmol	<sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
50		0.29
500		0.11
1000		0
3718		1.28

CH <sub>4</sub> concentration	
DEPTH(m)	nmol l <sup>-1</sup>
50	0.08
500	0.08
1000	0.05
1500	0.11
2000	0.08
2368	0.06
3318	0.09



EVENT #11      120390

<sup>3</sup>TdR accumulation

DEPTH(m)	nmol <sup>3</sup> TdR m <sup>-3</sup> hr <sup>-1</sup>
50	0.22
200	0.07
500	0.02
1000	0
3000	0.02

CH<sub>4</sub> concentration

DEPTH(m)	nmol l <sup>-1</sup>
50	0.08
200	0.06
500	0.20
1000	0.14
1500	0.10
2000	0.09
3000	0.04

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